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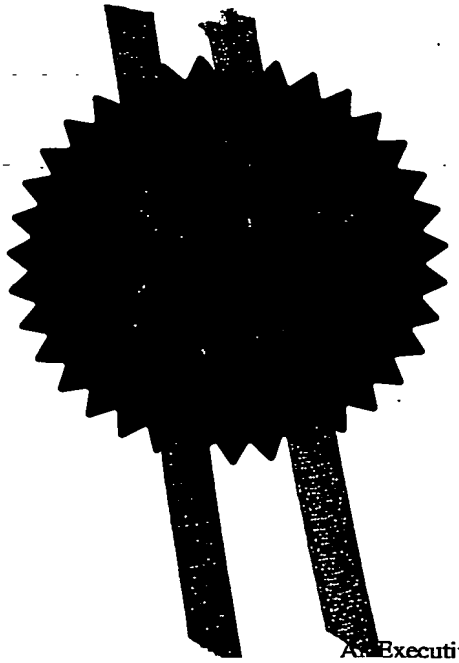

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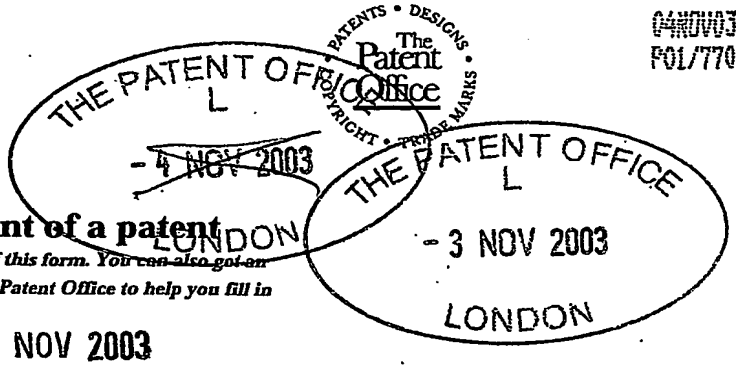

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P01/7700 0.00-0325624.5

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1. Your reference — N.89060 JHS

2. Patent application number *(The Patent Office will fill in this part)* — 0325624.5

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ISTITUTO SUPERIORE DI SANITA
Viale Regina Elena 299
00161 Roma
ITALY

Patents ADP number *(if you know it)* — 08691719001

If the applicant is a corporate body, give the country/state of its incorporation — ITALY

4. Title of the invention — USE OF MICROPARTICLES FOR ANTIGEN DELIVERY

5. Name of your agent *(if you have one)* — J.A. KEMP & CO.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

14 South Square
Gray's Inn
London
WC1R 5JJ

Patents ADP number *(if you know it)* — 00260001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

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a) any applicant named in part 3 is not an inventor, or
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Continuation sheets of this form	
Description	65
Claim(s)	2 DL
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Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	2 ✓
Request for preliminary examination and search *(Patents Form 9/77)*	1 ✓
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature J.A. KEMP & CO. Date 3 November 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

SEXTON, Jane Helen
020 7405 3292

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USE OF MICROPARTICLES FOR ANTIGEN DELIVERY

Field of the invention

The invention relates to the fields of antigen delivery and vaccines. More specifically, the invention relates to certain microparticles, and to antigen delivery and vaccine immunization strategies employing such microparticles. The invention in particular relates to microparticles that are useful in the prophylaxis and treatment of human immunodeficiency virus (HIV) infections.

Background of the invention

It is important that therapeutic or prophylactic peptides, and in particular vaccines, are efficiently delivered to their site of action without significant degradation. Polymeric microparticles encapsulating peptide antigens have been investigated as potential delivery systems for their capability to efficiently target the antigen to professional antigen-presenting cells and to release it in a controlled way over a prolonged period of time (O'Hagan DT., Recent advances in vaccine adjuvants for systemic and mucosal administration, J. Pharm. Pharmacol., 1998; 59:1-10; Nugent J, Wan Po L, Scott E., Design and delivery of non-parental vaccines, Review. J. Clin. Pharm. Therap., 1998; 23:257-85; and Alpar HO, Ward KR, Williamson ED., New strategies in vaccine delivery., S.T.P. Pharma. Sci., 2000; 10:269-78).

Although peptides encapsulated into a microparticulate matrix may be protected from unfavorable conditions encountered after parenteral or mucosal administration (Nedrud JG, Lamm ME., Adjuvants and the mucosal immune system, In: Spriggs DR, Koff WC, editors, Topics in vaccine adjuvant research, Boca Raton: CRC, 1991. p. 51-67), they often become unstable or are degraded. This may occur either during the encapsulation process, such as the exposure to organic solvents, high shear and freeze-drying, and/or in the body when the antigen is exposed to the low pH microenvironment caused by the degradation of the polymer (O'Hagan DT, Singh M, Gupta RK., Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines, Adv. Drug. Deliv. Rev. 1998; 32:225-46; and O'Hagan DT., supra).

Summary of the invention

The inventors have found that antigens may be fixed or adsorbed to the external surface of polymeric microparticles. Further the inventors have shown that these microparticles may be used to efficiently deliver antigens to target cells.

Accordingly the invention provides a microparticle comprising:

(a) a core which comprises a water insoluble polymer or copolymer, and

(b) a shell which comprises a hydrophilic polymer or copolymer and functional groups which are ionic or ionisable;

said microparticle having a disease-associated antigen adsorbed at the external surface.

The invention further provides:

- a method of production of a microparticle of invention;
- a pharmaceutical composition comprising a microparticle of the invention;
- a method of generating an immune response in an individual, said method comprising administering a microparticle of the invention in a therapeutically effective amount;
- a method of preventing or treating HIV infection or AIDS, said method comprising administering a microparticle of the invention in a therapeutically effective amount.
- a microparticle of the invention for use in a method of treatment of the human or animal body by therapy or diagnosis;
- use of a microparticle of the invention for the manufacture of a medicament for generating an immune response in an individual; and
- use of a microparticle of the invention for the manufacture of a medicament for preventing or treating HIV infection or AIDS.

Brief description of the Figures

Figure 1 shows BSA (•) and Trypsin (■) adsorption onto basic (HE1D; A) and acidic (H1D; B) microparticles.

Figure 2 shows analysis of Tat adsorption to the surface of acid polymeric microparticle by FACS analysis using an anti-Tat polyclonal rabbit serum. Two representative microparticles, A7, made of poly(styrene) and hemisuccinated polyvinyl alcohol (•) and 1E, constituted of poly(methyl methacrylate) and Eudragit L100-55 (■) are shown.

Figure 3 shows evaluation of cell proliferation in the presence of the microparticles alone or the Tat/microparticle complexes. HL3T1 cells were cultured for 96 h with 10 μg/ml (empty bars), 30 μg/ml (black bars), and 50 μg/ml (gray bars) of microparticles alone (A) or with the same doses of microparticles bound to Tat (1 μg/ml) (B). Controls were represented by untreated cells (None) or cells cultured with 1 μg/ml of Tat (Tat). Results are expressed as the mean (± S.D.) of sextuples.

Figure 4 shows murine macrophages phagocytosis of polymeric microparticles made of poly(styrene) and hemisuccinated poly(vinyl alcohol) and microparticles made of poly(methyl methacrylate) and Eudragit L100-55. Murine macrophages were cultured with microparticles, fixed, colored with toluidine blue and observed at a phase contrast microscope. Results are expressed as the percentage of cells that phagocytosed the microparticles.

Figure 5 shows analysis of microparticle uptake. Human monocytes (A), monocyte-derived dendritic cells (B), murine splenocytes (C) and HL3T1 cells (D) were cultured in the presence of fluorescent H1D microparticles for 24 h, fixed with paraformaldheyde and observed at fluorescent and confocal microscopes. Representative images of fluorescent microscopy are shown in panels A, B and C, and of confocal microscopy in panel D.

Figure 6 shows that polymeric microparticles deliver and release HIV-1 Tat intracellularly. HL3T1 cells were cultured in the presence of fluorescent-H1D (30 μg/ml) bound to Tat (5 μg/ml) (A) or with Tat alone (5 μg/ml) (B), fixed and analyzed by immunofluorescence using an anti-Tat monoclonal antibody. For the same microscopic field, green (H1D), red (Tat), blue (DAPI) and phase contrast (cells) images were taken with a CCD camera and overlapped with a Adobe Photoshop program.

Figure 7 shows that polymeric microparticles protect HIV-1 Tat from oxidation. HL3T1 cells, containing an integrated copy of the reporter vector HIV-1 LTR-CAT, were incubated with Tat (1 μg/ml) adsorbed to the microparticles (30 μg/ml) and exposed to air and light for 16 h at room temperature. Control cells were incubated with the same dose of the protein, which was untreated (Tat) or oxidized by exposure to air and light (Tat ox). The percentage of CAT activity was calculated as described (Betti *et al.*, Vaccine, 2001; 19:3408-3419). Results are the mean of two independent experiments.

Figure 8 shows analysis of the expression of the HIV-1 Tat protein bound to polymeric microparticles made of poly(styrene) and hemisuccinated poly(vinyl alcohol)

(A4, A7) and of poly(methyl methacrylate) and Eudragit L100-55 (1D, 1E and H1D). HL3T1 cells were incubated with increasing amounts of Tat alone and with the same amounts of Tat bound to each microparticle (30 μg/ml). CAT activity was measured 48 hours later. Results are the mean of three independent experiments.

Figures 9 shows analysis of γIFN released from splenocytes of mice vaccinated, at weeks 0 and 4, with Tat/microparticle complexes. Splenocytes, obtained two weeks after the second immunization, were pooled by treatment groups, and co-cultured with BALB/c 3T3-Tat expressing cells in the presence of Tat for four days. Results are expressed as pg/ml of γIFN released in culture supernatants.

Figure 10 shows analysis of T cell proliferation (left panels) and of γIFN release (right panels) in response to Tat-derived 15-mer peptides delivered as A4/Tat (A), H1D/Tat (B) or just Tat (C). Splenocytes of mice, immunized at weeks 0 and 4 and sacrificed two weeks after the second immunization, were pooled by treatment groups and co-cultured for four days with BALB/c 3T3-Tat expressing cells in the presence of Tat. After Ficoll purification, cells were cultured with irradiated naive splenocytes pulsed with Tat peptides, and with or without PHA. γIFN release on culture supernatants and T-cell ^{3}[H] thymidine incorporation were measured, respectively, after 24 and 96 hours of culture. Only the results to reactive peptides are shown and they are expressed as fold increase of ^{3}[H] thymidine incorporation and release as compared to values of the same cultures grown without PHA.

Figure 11 shows histologic examination of the inflammatory reactions present at the site of inoculation. Two representative mice received an intramuscular injection with Tat (2 μg) adsorbed to A7 microparticles (A, C) and Tat (2 μg) in Freund's adjuvant (B, D) at weeks 0, 4, and 8. A7-Tat inoculation caused a scarce inflammatory reaction (A) in the muscle fibres consisting exclusively of macrophages (C). Tat plus Freund inoculation induced an intense inflammatory reaction prevalently in the adipose tissue surrounding the muscle fibers with presence of macrophages and clear lacunae of lipolysis (B) and in some cases with extensive necrosis constituted by amorphous material and nuclear debris (D). Hematoxylin-eosin staining; A and B: 40X; C: 400X; and D: 200X.

Brief description of the Sequence listing

SEQ ID NO: 1 shows the nucleotide sequence that encodes the full length. HIV-1 Tat protein from HTLV-III, BH10 CLONE, CLADE B. This is the parent sequence for the

TC peptides (SEQ ID NOs: 33 to 48).

SEQ ID NO: 2 shows the 102 amino acid sequence of full length HIV-1 Tat protein from HILV, BH10 CLONE CLADE B.

SEQ ID NOs: 3 to 32 show the nucleotide and amino acid sequences of variants of the full length HIV-1 Tat protein isolated from HTLV-III, BH10 CLONE, CLADE B. The length and sequence of Tat varies depending on the viral isolate.

SEQ ID NO: 3 shows the nucleotide sequence that encodes the shorter version of HIV-1 Tat protein (BH10).

SEQ ID NO: 4 shows the 86 amino acid shorter version of HIV-1 Tat protein (BH10). This sequence corresponds to residues 1 to 86 of SEQ ID NO: 1.

SEQ ID NO: 5 shows the nucleotide sequence that encodes the cysteine 22 mutant of BH10 (SEQ ID NO: 4).

SEQ ID NO: 6 shows the 86 amino acid cysteine 22 mutant of BH10 (SEQ ID NO: 4).

SEQ ID NO: 7 shows the nucleotide sequence that encodes the lysine 41 mutant of BH10 (SEQ ID NO: 4).

SEQ ID NO: 8 shows the 86 amino acid lysine 41 mutant of BH10 (SEQ ID NO: 4).

SEQ ID NO: 9 shows the nucleotide sequence that encodes the RGDΔ mutant of BH10 (SEQ ID NO: 4).

SEQ ID NO: 10 shows the 83 amino acid RGDΔ mutant of BH10 (SEQ ID NO: 4).

SEQ ID NO: 11 shows the nucleotide sequence that encodes the lysine 41 RGDΔ mutant of BH10 (SEQ ID NO: 4).

SEQ ID NO: 12 shows the 83 amino acid lysine 41 RGDΔ mutant of BH10 (SEQ ID NO: 4).

SEQ ID NO: 13 shows the nucleotide sequence that encodes the consensus_ A-A1-A2 variant of HIV-1 Tat protein.

SEQ ID NO: 14 shows the 101 amino acid consensus_A-A1-A2 variant of HIV-1 Tat protein.

SEQ ID NO: 15 shows the nucleotide sequence that encodes the consensus_B variant of HIV-1 Tat protein.

SEQ ID NO: 16 shows the 101 amino acid consensus_B variant of HIV-1 Tat protein.

SEQ ID NO: 17 shows the nucleotide sequence that encodes the consensus_C variant of HIV-1 Tat protein.

SEQ ID NO: 18 shows the 101 amino acid consensus_C variant of HIV-1 Tat protein.

SEQ ID NO: 19 shows the nucleotide sequence that encodes the consensus_D variant D of HIV-1 Tat protein.

SEQ ID NO: 20 shows the 86 amino acid consensus_D variant of the HIV-1 Tat protein.

SEQ ID NO: 21 shows the nucleotide sequence that encodes the consensus_ F1-F2 variant of HIV-1 Tat protein.

SEQ ID NO: 22 shows the 101 amino acid consensus_F1-F2 variant of HIV-1 Tat protein.

SEQ ID NO: 23 shows the nucleotide sequence that encodes the consensus_G variant of the HIV-1 Tat protein.

SEQ ID NO: 24 shows the 101 amino acid consensus_G variant of the HIV-1 Tat protein.

SEQ ID NO: 25 shows the nucleotide sequence that encodes the consensus_H variant of the HIV-1 Tat protein.

SEQ ID NO: 26 shows the 86 amino acid consensus_H variant of the HIV-1 Tat protein.

SEQ ID NO: 27 shows the nucleotide sequence that encodes the consensus_CRF01 variant of the HIV-1 Tat protein.

SEQ ID NO: 28 shows the 101 amino acid consensus_CRF01 variant of the HIV-1 Tat protein.

SEQ ID NO: 29 shows the nucleotide sequence that encodes the consensus_ CRF01 variant of the HIV-1 Tat protein.

SEQ ID NO: 30 shows the nucleotide sequence that encodes the consensus_ CRF02 variant of the HIV-1 Tat protein.

SEQ ID NO: 31 shows the 101 amino acid consensus_CRF02 of the HIV-1 Tat protein.

SEQ ID NO: 31 shows the nucleotide sequence that encodes the consensus_O variant of HIV-1 Tat protein.

SEQ ID NO: 32 shows the 115 amino acid consensus_O variant of the HIV-1 Tat

protein.

SEQ ID NO: 33 shows the sequence of the TC27 peptide in Table 2.

SEQ ID NO: 34 shows the sequence of the TC28 peptide in Table 2.

SEQ ID NO: 35 shows the sequence of the TC29 peptide in Table 2.

SEQ ID NO: 36 shows the sequence of the TC30 peptide in Table 2.

SEQ ID NO: 37 shows the sequence of the TC31 peptide in Table 2.

SEQ ID NO: 38 shows the sequence of the TC32 peptide in Table 2.

SEQ ID NO: 39 shows the sequence of the TC33 peptide in Table 2.

SEQ ID NO: 40 shows the sequence of the TC34 peptide in Table 2.

SEQ ID NO: 41 shows the sequence of the TC35 peptide in Table 2.

SEQ ID NO: 42 shows the sequence of the TC36 peptide in Table 2.

SEQ ID NO: 43 shows the sequence of the TC37 peptide in Table 2.

SEQ ID NO: 44 shows the sequence of the TC38 peptide in Table 2.

SEQ ID NO: 45 shows the sequence of the TC39 peptide in Table 2.

SEQ ID NO: 46 shows the sequence of the TC40 peptide in Table 2.

SEQ ID NO: 47 shows the sequence of the TC41 peptide in Table 2.

SEQ ID NO: 48 shows the sequence of the TC42 peptide in Table 2.

Detailed description of the invention

It is to be understood that this invention is not limited to particular antigens. It is also to be understood that different applications of the disclosed methods may be tailored to the specific needs in the art. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

In addition as used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more such agents, reference to "a microparticle" includes reference to mixtures of two or more microparticles, reference to "a target" cell" includes two or more such cells, and the like.

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

The invention provides microparticles for delivering antigens to target cells. The microparticles have an antigen adsorbed or fixed onto their external surface. The term

"microparticle of the invention" is herein defined as a microparticle with an antigen adsorbed at the external surface.

The microparticles comprise: a core which comprises a water insoluble polymer or copolymer; and a shell which comprises a hydrophilic polymer or copolymer and functional groups which are ionic or ionisable. The microparticles are typically obtainable by dispersion polymerization of a water-insoluble monomer in the presence of a hydrophilic polymer or copolymer. The water-insoluble monomer is polymerized to form the core and the hydrophilic polymer or copolymer forms the shell. The outer shell is typically covalently bonded to the inner core. The external microparticle surface is typically a hydrophilic shell that comprises ionic or ionisable chemical groups. The microparticle surface has an overall positive or negative charge. The microparticles are cationic or anionic. The microparticles preferably have a net positive or negative charge over their entire external surface. The surface charge density typically varies across the surface of the microparticles.

The shell and core of the microparticles are preferably composed of a biocompatible polymeric material. The term "biocompatible polymeric material" is defined as a polymeric material which is not toxic to an animal and not carcinogenic. The matrix material may also be biodegradable in the sense that the polymeric material should degrade by bodily processes *in vivo* to products readily disposable by the body and should not accumulate in the body. On the other hand, where the microparticle is being inserted into a tissue which is naturally shed by the organism (eg. sloughing of the skin), the matrix material need not be biodegradable.

Suitable water insoluble polymer forming materials for use in the core of the microparticles include, but are not limited to, poly(dienes) such as poly(butadiene) and the like; poly(alkenes) such as polyethylene, polypropylene, and the like; poly(acrylics) such as poly(acrylic acid) and the like; poly(methacrylics) such as poly(methyl methacrylate), poly(hydroxyethyl methacrylate), and the like; poly(vinyl ethers); poly(vinyl alcohols); poly(vinyl ketones); poly(vinyl halides) such as poly(vinyl chloride) and the like; poly(vinyl nitriles), poly(vinyl esters) such as poly(vinyl acetate) and the like; poly(vinyl pyridines) such as poly(2-vinyl pyridine), poly(5-methyl-2-vinyl pyridine) and the like; poly(styrenes); poly(carbonates); poly(esters); poly(orthoesters); poly(esteramides); poly(anhydrides); poly(urethanes); poly(amides); cellulose ethers such as methyl cellulose, hydroxyethyl cellulose, hydroxypropyl methyl cellulose, and the like; cellulose esters such

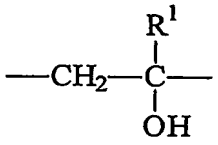
as cellulose acetate, cellulose acetate phthalate, cellulose acetate butyrate, and the like; poly(saccharides), proteins, gelatin, starch, gums, resins, and the like. The polymeric materials may be cross-linked.

Preferred materials include, but are not limited to, polyacrylates, polymethacrylates and polystyrenes. The term "poly(meth)acrylate" as used herein encompasses both polyacrylates and polymethacrylates. Likewise the term "(meth)acrylate" encompasses both acrylates and methacrylates.

Preferred poly(meth)acrylates which may be used as core materials include poly(alkyl (meth)acrylates), in particular poly(C_{1-10} alkyl (meth)acrylates), and preferably poly(C_{1-6} alkyl (meth)acrylates) such as poly(methyl acrylate), poly(methyl methacrylate), poly(ethyl acrylate), and poly(ethyl methacrylate). Poly(methyl methacrylate) (PMMA) is especially preferred as the core material.

Suitable hydrophilic polymer forming materials for use in the hydrophilic shell of the microparticles include, but are not limited to, hemisuccinated polyvinylalcohols and Eudragit® copolymers.

A preferred material for the hydrophilic shell is a polymer or copolymer which comprises repeating units of formula I:

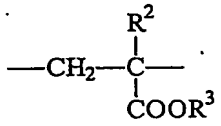


(I)

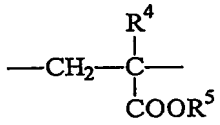
wherein R1 is hydrogen, methyl or ethyl.

The hydrophilicity may be augmented by reacting this polymer with a diacid such as maleic or succinic acid. A particularly preferred hydrophilic polymer is hemisuccinated polyvinylalcohol.

Another preferred class of hydrophilic polymer that may be used in the hydrophilic shell of the microparticles is a copolymer which comprises repeating units of formulae (II) and (III):



(II)



(III)

wherein R^2 and Reach independently represent hydrogen or methyl, R^3 represents hydrogen, -A-NR^9R^{10} or -A-$N^+R^9R^{10}R^{11}$ X^-, in which A represents C_{1-10} alkylene, R^9, $R^{10}$and R^{11} each independently represent hydrogen or C_{1-10} alkyl and X represents halogen, and R^5 represents C_{1-10} alkyl.

In a particular embodiment, R^2 in the repeating unit of formula (II) is hydrogen or methyl. A in the monomer of formula (II) is C_{1-10} alkylene and is preferably a C_{1-6} alkylene group, for example a methylene, ethylene, propylene, butylene, pentylene or hexylene group or isomer thereof. Ethylene is preferred.

R^9 in the monomer of formula (II) is hydrogen or C_{1-10} alkyl, and is preferably a C_{1-10} alkyl group, more preferably a C_{1-6} alkyl group, for example a methyl, ethyl, propyl, i-propyl, –butyl, sec-butyl or tert-butyl group, or a pentyl or hexyl group or isomer thereof. Methyl and ethyl are preferred, particularly methyl.

R^{10} in the monomer of formula (II) is hydrogen or C_{1-10} alkyl, and is preferably a C_{1-10} alkyl group, more preferably a C_{1-6} alkyl group, for example a methyl, ethyl, propyl, i-propyl, n-butyl, sec-butyl or tert-butyl group, or a pentyl or hexyl group or isomer thereof. Methyl and ethyl are preferred, particularly methyl.

R^{11} in the monomer of formula (II) is hydrogen or C_{1-10} alkyl, and is preferably a C_{1-10} alkyl group, more preferably a C_{1-6} alkyl group, for example a methyl, ethyl, propyl, i-propyl, n-butyl, sec-butyl or tert-butyl group, or a pentyl or hexyl group or isomer thereof. Methyl and ethyl are preferred, particularly methyl.

R^4 in the repeating unit of formula (III) is hydrogen or methyl.

R^5 in the repeating unit of formula (III) is C_{1-10} alkyl, and is preferably a C_{1-6} alkyl group, for example a methyl, ethyl, propyl, i-propyl, n-butyl, sec-butyl or tert-butyl group,

or a pentyl or hexyl group or isomer thereof. Methyl, ethyl and butyl are preferred.

The halogen atom represented by X in repeating unit of formula (II) is fluorine, chlorine, bromine or iodine, preferably bromine or iodine, most preferably bromine.

An example of a copolymer comprising repeating units of formulae (II) and (III) which may be used in the present invention is a copolymer of methacrylic acid and ethyl acrylate, for example a statistical copolymer in which the ratio of the free carboxyl groups to the ester groups is approximately 1:1. A suitable copolymer is commercially available from Röhm Pharma under the trade name Eudragit® L 100-55.

A further example of a copolymer comprising repeating units of formulae (II) and (III) which may be used in the present invention is a copolymer of 2-(dimethylamino)ethyl methacrylate and C_{1-6} alkyl methacrylate, for example a copolymer of 2-(dimethylamino)ethyl methacrylate, methyl methacrylate and butyl methacrylate. A suitable copolymer is commercially available from Röhm Pharma under the trade name Eudragit® E 100.

The hydrophilic polymer forming materials contain chemical groups that are ionic or ionisable. Preferably these groups are ionic or ionisable at physiological pH. The term "physiological pH" refers to the pH in the blood and extracellular fluid of an individual. The physiological pH is typically from 7.2 to 7.6 and preferably 7.4.

These water insoluble and hydrophilic polymeric materials may be used alone, as physical mixtures (blends) or as copolymers (which may be block copolymers). Again, these polymers may be cross-linked. The copolymers may be block, random or regular copolymers.

Usually, a satisfactory number-average molecular weight is in the range of 5,000 to 500,000 daltons, more preferably in the range of 10,000 to 500,000 daltons. The polymers mentioned above generally have number-average molecular weights of from 30,000 to 50,000 daltons, up to about 120,000 daltons such as from 80,000 to 100,000 daltons. A person skilled in the art would understand the appropriate number-average molecular weight range for a specific polymer.

Conventional methods for the construction of microparticles may be used to construct the microparticles of the invention. The microparticles are obtainable by dispersion polymerization of monomers. This method is described in Sparnacci *et al.* Macromolecular Chemistry and Physics, 2002: 203 (10-11): 1364-1369. Polymers are formed by the polymerization of one monomer. Copolymers are formed by the

polymerization of more than one monomer. Thus one or more water insoluble core monomers may be included in the polymerization reaction. Thus one or more hydrophilic shell polymers may be included in the polymerization reaction.

Typically, the core monomer, shell polymer and a radical initiator are dissolved in a suitable solvent under a nitrogen atmosphere. Suitable solvents include organic solvents such as acetone, halogenated hydrocarbons such as chloroform, methylene chloride and the like, aromatic hydrocarbon compounds, halogenated aromatic hydrocarbon compounds, cyclic ethers, alcohols, ethyl acetate and the like. Preferred solvents are methanol, ethanol, a 1:1 ratio mixture of ethanol and 2-methoxyethanol and a mixture of methanol and water (in a ratio between 7:3 and 9:1). The mixture of materials in the solvent may undergo freeze thaw cycles depending on the polymeric materials used. The temperature during the formation of the dispersion is not especially critical but can influence the size and quality of the microparticles. Moreover, depending on the solvent employed, the temperature must not be too low or the solvent and processing medium will solidify or the processing medium will become too viscous for practical purposes, or too high that the processing medium will evaporate, or that the liquid processing medium will not be maintained. Accordingly, the dispersion process can be conducted at any temperature which maintains stable operating conditions, which preferred temperature being about 30°C to 80°C, depending upon the materials selected.

The dispersed microparticles may be isolated from the solvent by any convenient means of separation. Thus, for example, the reaction mixture may undergo several rounds of centrifugation and redispersion with the solvent followed by several rounds of centrifugation and redispersion in water.

Following the isolation of the microparticles from the dispersion solvent, the microparticles may be dried by exposure to air or by other conventional drying techniques such as lyophilization, vacuum drying, drying over a desiccant, or the like. Prior to absorption the microparticles may be redispersed in a suitable liquid and temporarily stored. The skilled person will recognise under what conditions the microparticles of the invention may be stored. Typically, the microparticles are stored at a low temperature, for example 4°C.

The microparticles usually have a spherical shape, although irregularly-shaped microparticles are possible. When viewed under a microscope, therefore, the particles are typically spheroidal but may be elliptical, irregular in shape or toroidal. The microparticles

vary in size, ranging from 0.1μm to 10 μm, typically from 0.5μm or 0.75 μm to 4 μm, or typically from 1μm, 1.5μm or 2.5 μm to 6 μm. The maximum size is the diameter in spherical microparticles.

The size of the microparticles can be measured using conventional techniques such as microscopic techniques (where particles are sized directly and individually rather than grouped statistically), absorption of gasses, or permeability techniques. If desired, automatic particle-size counters can be used (for example, the Coulter Counter, HIAC Counter, or Gelman Automatic Particle Counter) to ascertain average particle size.

Actual microparticle density can be readily ascertained using known quantification techniques such as helium pycnometry and the like. Alternatively, envelope ("tap") density measurements can be used to assess the density of a particulate composition. Envelope density information is particularly useful in characterizing the density of objects of irregular size and shape. Envelope density, or "bulk density," is the mass of an object divided by its volume, where the volume includes that of its pores and small cavities. Other, indirect methods are available which correlate to density of individual particles. A number of methods of determining envelope density are known in the art, including wax immersion, mercury displacement, water absorption and apparent specific gravity techniques. A number of suitable devices are also available for determining envelope density, for example, the GeoPyc™ Model 1360, available from the Micromeritics Instrument Corp. The difference between the absolute density and envelope density of a sample pharmaceutical composition provides information about the sample's percentage total porosity and specific pore volume.

Microparticle morphology, particularly the shape of a particle, can be readily assessed using standard light or electron microscopy. It is preferred that the particles have a substantially spherical or at least substantially spherical shape. It is also preferred that the particles have an axis ratio of 2 or less, i.e. from 2:1 to 1:1, to avoid the presence of rod- or needle-shaped particles. These same microscopic techniques can also be used to assess the particle surface characteristics, for example, the amount and extent of surface voids or degree of porosity.

In an especially preferred embodiment, the microparticles comprise a core of poly(styrene) and a hydrophilic shell of hemisuccinated poly(vinyl alcohol) and have an average size of from 0.9 μm to 4 μm. In another especially preferred embodiment, the microparticles comprise a core of poly(methyl methacrylate) and a hydrophilic shell of

Eudragit® E100 and have a average size from 1.5 μm to 8.5 μm. In a further especially preferred embodiment, the microparticles comprise a core of poly(methyl methacrylate) and a hydrophilic shell of Eudragit® L100/55 and have an average size from 1.5 μm to 2.0 μm.

The term "adsorbed" or "fixed" means that the microbial antigen is attached to the external surface of the shell of the microparticle. The absorption or fixation preferably occurs by electrostatic attraction. Electrostatic attraction is the attraction or bonding generated between two or more ionic or ionisable chemical groups which are oppositely charged. The absorption or fixation is typically reversible.

The antigen preferably has a net charge that attracts it to the ionic hydrophilic shell of the microparticle. The antigen typically has one or more charged chemical or ionic groups. In the case of the antigen being a peptide, the antigen typically has one or more charged amino acid residues. The antigen typically has a net positive or negative charge. The antigen preferably has a net charge that is opposite to the charge of the hydrophilic shell of the microparticle. As a result, basic antigens may be adsorbed onto acid microparticles and acidic antigens may be adsorbed onto basic microparticles.

The antigen may be adsorbed onto the microparticles by mixing a solution of the antigen with a liquid suspension of the microparticles. The antigen and microparticles are typically mixed in a suitable liquid, for example a physiological buffer such as phosphate buffered saline (PBS). The mixture may be left for sometime under conditions suitable for the preservation of the antigen and formation of the bond between the antigen and microparticles. These conditions will be recognised by a person skilled in the art. Adsorption is preferably carried out at 0° to 37°C, preferably 4 to 25°C and in the dark. Adsorption is typically carried out for from 30 and 180 minutes. Following adsorption, the microparticles of the invention may be separated from the adsorption liquid by methods known in the art, for example centrifugation. The microparticle-antigen complexes may then be resuspended in a liquid suitable for administration to an individual.

The term"disease-associated antigen" is used in it broadest sense to refer to any antigen associated with a disease. An antigen is a molecule which contains one or more epitopes that will stimulate a host's immune system to make a cellular antigen-specific immune response, and/or a humoral antibody response. Thus, a disease-associated antigen is a molecule which contains epitopes that will stimulate a host's immune system to make a cellular antigen-specific immune response and/or a humoral antibody response against

the disease. The disease-associated antigen may therefore be used for prophylactic or therapeutic purposes.

Disease-associated antigens are preferably associated with infection by microbes, typically microbial antigens, or associated with cancer, typically tumours. Thus, antigens that may be used in the invention include proteins, polypeptides, immunogenic protein fragments, oligosaccharides, polysaccharides, and the like. The term "immunogenic fragment" means a fragment of any antigen described herein that itself is capable of stimulating a host's immune system to make a cellular antigen-specific immune response and/or a humoral antibody response.

The disease-associated antigen may be associated with microbial infection and thus contain epitopes that will stimulate a host's immune system to make a cellular antigen-specific immune response and/or a humoral antibody response against the microbial infection. The antigen is typically found in the body of an individual when that individual has a microbial infection. The antigen is preferably derived from a microbe, namely microbial. Thus, the antigen may be derived from any known microbe, for example, virus, bacterium, parasites, protists such as protozoans, or fungus, and can be a whole organism or immunogenic parts thereof, for example, cell wall components.

Antigens for use in the invention include, but are not limited to, those containing, or derived from, members of the families Picornaviridae (for example, polioviruses, etc.); Caliciviridae; Togaviridae (for example, rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabodoviridae (for example, rabies virus, measels virus, respiratory syncytial virus, etc.); Orthomyxoviridae (for example, influenza virus types A, B and C, etc.); Bunyaviridae; Arenaviridae; Retroviradae (for example, HTLV-I; HTLV-II; HIV-1; and HIV-2); simian immunodeficiency virus (SIV) among others. Additionally, viral antigens may be derived from a papilloma virus (for example, HPV); a herpes virus, i.e. herpes simplex 1 and 2; a hepatitis virus, for example, hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis D virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV) and the tick-borne encephalitis viruses; smallpox, parainfluenza, varicella-zoster, cytomeglavirus, Epstein-Barr, rotavirus, rhinovirus, adenovirus, papillomavirus, poliovirus, mumps, rubella, coxsackieviruses, equine encephalitis, Japanese encephalitis, yellow fever, Rift Valley fever, lymphocytic choriomeningitis, and the like. See for example, Virology, 3rd Edition(W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and

D.M. Knipe, eds. 1991), for a description of these and other viruses.

Bacterial antigens include, but are not limited to, those containing or derived from organisms that cause diphtheria, cholera, tuberculosis, tetanus, pertussis, meningitis, and other pathogenic states, including *Meningococcus* A, B and C, *Hemophilus influenza* type B (HIB), and *Helicobacter pylori, Streptococcus pneumoniae, Staphylococcus aureus, Streptococcus pyrogenes, Corynebacterium diphtheriae, Listeria monocytogenes, Bacillus anthracis, Clostridium tetani, Clostridium botulinum, Clostridium perfringens, Neisseria meningitidis, Neisseria gonorrhoeae, Streptococcus mutans, Pseudomonas aeruginosa, Salmonella typhi, Haemophilus parainfluenzae, Bordetella pertussis, Francisella tularensis, Yersinia pestis, Vibrio cholerae, Legionella pneumophila, Mycobacterium tuberculosis, Mycobacterium leprae, Treponema pallidum, Leptspirosis interrogans, Borrelia burgdorferi, Campylobacter jejuni*, and the like.

Examples of anti-parasitic antigens include, but are not limited to, those derived from organisms causing malaria and Lyme disease. Antigens of such fungal, protozoan, and parasitic organisms such as *Cryptococcus neoformans, Histoplasma capsulatum, Candida albicans, Candida tropicalis, Nocardia asteroides, Rickettsia ricketsii, Rickettsia typhi, Mycoplasma pneumoniae, Chlamydial psittaci, Chlamydial trachomatis, Plasmodium falciparum, Trypanosoma brucei, Entamoeba histolytica, Toxoplasma gondii, Trichomonas vaginalis, Schistosoma mansoni*, and the like.

In a especially preferred embodiment, the antigen adsorbed on the microparticle is the HIV Tat protein (SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32) or an immunogenic fragment thereof.

The disease-associated antigen may be cancer-associated. A cancer-associated antigen is a molecule which contains epitopes that will stimulate a host's immune system to make a cellular antigen-specific immune response and/or a humoral antibody response against the cancer. A cancer-associated antigen is typically found in the body of an individual when that individual has cancer. A cancer-associated antigen is preferably derived from a tumor. Cancer-associated antigens include, but are not limited to, cancer-associated antigens (CAA), for example, CAA-breast, CAA-ovarian and CAA-pancreatic; the melanocyte differentiation antigens, for example, Melan A/MART-1, tyrosinase and gp100; cancer-germ cell (CG) antigens, for example, MAGE and NY-ESO-1; mutational antigens, for example, MUM-1, p53 and CDK-4; over-expressed self-antigens, for example, p53 and HER2/NEU and tumor proteins derived from non-primary open reading

frame mRNA sequences, for example, LAGE1.

Synthetic antigens are also included in the definition of antigen, for example, haptens, polyepitopes, flanking epitopes, and other recombinant or recombinant or synthetically derived antigens (Bergmann et al. (1993) Eur. J. Immunol. 23:2777-2781; Bergmann et al. (1996) J. Immunol. 157:3242-3249; Suhrbier, A. (1997) Immunol. and Cell Biol. 75:402-408; Gardner et al. (1998) 12th World AIDS Conference, Geneva, Switzerland (June 28 – July 3, 1998). A synthetic disease-associated antigen is a synthetic molecule which contains epitopes that will stimulate a host's immune system to make a cellular antigen-specific immune response and/or a humoral antibody response against the disease.

The antigen or immunogenic fragments of antigens mentioned herein typically comprise one or more T cell epitopes. "T cell epitopes" are generally those features of a peptide structure capable of inducing a T cell response. In this regard, it is accepted in the art that T cell epitopes comprise linear peptide determinants that assume extended conformations within the peptide-binding cleft of MHC molecules (Unanue et al. (1987) Science 236: 551-557). As used herein, a T cell epitope is generally a peptide having about 8-15, preferably 5-10 or more amino acid residues.

The microparticles of the invention can be viewed as a "vaccine composition" and as such includes any pharmaceutical composition which contains an antigen and which can be used to prevent or treat a disease or condition in a subject. The term encompasses both subunit vaccines, i.e., vaccine compositions containing antigens which are separate and discrete from a whole organism with which the antigen is associated in nature, as well as compositions containing whole killed, attenuated or inactivated bacteria, viruses, parasites or other microbes. The vaccine can also comprise a cytokine that may further improve the effectiveness of the vaccine.

The microparticles of the invention can comprise from about 1 to about 99% of the antigen by weight, for example from about 0.01 to about 10% of the antigen by weight. The microparticles can therefore comprise from 0.05 to 10% of the antigen by weight such as from 2 to 8% of the antigen by weight or from 5 to 6% of the antigen by weight. The actual amount depends on a number of factors include the nature of the antigen, the dose desired and other variables readily appreciated by those skilled in the art.

The inventors have shown that administration of microparticles of the invention generates an immune response in an individual. Thus the inventors have shown that

adsorption of the antigen to the external surface of the microparticle preserves the biological activity of the antigen. Thus the inventors have also shown that the adsorption of the antigen to the microparticle does not affect the immunogenicity of the antigen. The inventors have also shown that adsorption of the antigen to the microparticle reduce the amount of antigen required to generate an immune response, eliminates or reduces the number of antigen booster shots needed and improves the handling or shelf-life of the antigen.

Accordingly, the present invention also relates to prophylactic or therapeutic methods utilising the microparticles of the invention. These prophylactic or therapeutic methods involve generating an immune response in an individual using the microparticles of the invention. Thus, the microparticles of the invention may be administered to an individual to generate an immune response in that individual. Alternatively, the microparticles may be used in the manufacture of a medicament for generating an immune response in an individual.

The term "administer" or "deliver" is intended to refer to any delivery method of contacting the microparticles with the target cells or tissue. The term "tissue" refers to the soft tissues of an animal, patient, subject etc. as defined herein, which term includes, but is not limited to, skin, mucosal tissue (eg. buccal, conjunctival, gums), vaginal and the like. Bone may however be treated too by the particles of the invention, for example bone fractures.

When administration is for the purpose of treatment, administration may be either for prophylactic or therapeutic purpose. When provided prophylactically, the antigen is provided in advance of any symptom. The prophylactic administration of the antigen serves to prevent or attenuate any subsequent symptom. When provided therapeutically the antigen is provided at (or shortly after) the onset of a symptom. The therapeutic administration of the antigen serves to attenuate any actual symptom. Administration and therefore the methods of the invention may be carried out *in vivo* or *in vitro*.

The terms "animal", "individual", "patient" and "subject" are used interchangeably herein to refer to a subset of organisms which include any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as bovine animals, for example cattle; ovine animals, for example sheep; porcine, for example pigs; rabbit, goats and horses; domestic mammals such as dogs and cats; wild animals;

laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese; and the like. The terms do not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. In one embodiment, the individual is typically capable of being infected by HIV.

The invention includes treating a disease state in an animal by administering the microparticles described herein to a subject in need of such treatment. As used herein, the term "treatment" or "treating" includes any of the following: the prevention of infection or reinfection; the reduction or elimination of symptoms; and the reduction or complete elimination of a pathogen. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection). The methods of this invention also include effecting a change in an organism by administering the microparticles.

The methods of the invention may be carried out on individuals at risk of disease associated with antigen. Typically, the methods of the invention are carried out on individuals at risk of microbial infection or cancer associated with or caused by the antigen. In a preferred embodiment, the method of the invention is carried out on individuals at risk of infection with HIV or developing AIDS.

The methods described herein elicit an immune response against particular antigens for the treatment and/or prevention of a disease and/or any condition which is caused by or exacerbated by the disease. The methods described herein typically elicit an immune response against particular antigens for the treatment and/or prevention of microbial infection or cancer and/or any condition which is caused by or exacerbated by microbial infection or cancer. In a particular embodiment, the methods described herein elicit an immune response against particular antigens for the treatment and/or prevention of HIV infection and/or any condition which is caused by or exacerbated by HIV infection, such as AIDS.

The method of the invention is carried out for the purpose of stimulating a suitable immune response. By suitable immune response, it is meant that the method can bring about in an immunized subject an immune response characterized by the increased production of antibodies and/or production of B and/or T lymphocytes specific for an antigen, wherein the immune response can protect the subject against subsequent infection. In a preferred embodiment, the method can bring about in an immunized subject an immune response characterized by the increased production of antibodies and/or

production of B and/or T lymphocytes specific for HIV-1 Tat, wherein the immune response can protect the subject against subsequent infection with homologous or heterologous strains of HIV, reduce viral burden, bring about resolution of infection in a shorter amount of time relative to a non-immunized subject, or prevent or reduce clinical manifestation of disease symptoms, such as AIDS symptoms.

The aim of the method of the invention is to generate an immune response in an individual. Preferably, antibodies to the antigen are generated in the individual. Preferably IgG antibodies to the antigen are generated. Antibody responses may be measured using standard assays such as radioimmunoassay, ELISAs and the like, well known in the art.

Preferably cell-mediated immunity is generated, and in particular a CD8 T cell response generated. In this case the administration of the microparticles may, for example increases the level of antigen experienced CD8 T cells. The CD8 T cell response may be measured using any suitable assay (and thus may be capable of being detected in such an assay), such as an ELISPOT assay, preferably an γIFN-ELISPOT assay, CD8 proliferation to peptides and CTL assays. Preferably, a CD4 T cell response is also generated, such as the CD4 Th1 response. Thus the levels of antigen experienced CD4 T cells may also be increased. Such increased levels of CD4 T cells may be detected using a suitable assay, such as a proliferation assay.

The invention further provides the microparticles of the invention, namely microparticles with adsorbed antigens, in a pharmaceutical composition which also includes a pharmaceutically acceptable excipient. Such an "excipient" generally refers to a substantially inert material that is nontoxic and does not interact with other components of the composition in a deleterious manner.

These excipients, vehicles and auxiliary substances are generally pharmaceutical agents that do not themselves induce an immune response in the individual receiving the composition, and which may be administered without undue toxicity.

Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, polyethyleneglycol, hyaluronic acid, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulphates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

It is also preferred, although not required, that an antigen composition will contain a pharmaceutically acceptable carrier that serves as a stabilizer, particularly for peptide,

protein or other like antigens. Examples of suitable carriers that also act as stabilizers for peptides include, without limitation, pharmaceutical grades of dextrose, sucrose, lactose, trehalose, mannitol, sorbitol, inositol, dextran, and the like. Other suitable carriers include, again without limitation, starch, cellulose, sodium or calcium phosphates, citric acid, tartaric acid, glycine, high molecular weight polyethylene glycols (PEGs), and combination thereof. It may also be useful to employ a charged lipid and/or detergent. Suitable charged lipids include, without limitation, phosphatidylcholines (lecithin), and the like. Detergents will typically be a nonionic, anionic, cationic or amphoteric surfactant. Examples of suitable surfactants include, for example, Tergitol® and Triton® surfactants (Union Carbide Chemicals and Plastics, Danbury, CT), polyoxyethylenesorbitans, for example, TWEEN® surfactants (Atlas Chemical Industries, Wilmington, DE), polyoxyethylene ethers, for example Brij, pharmaceutically acceptable fatty acid esters, for example, lauryl sulfate and salts thereof (SDS), and like materials.

A thorough discussion of pharmaceutically acceptable excipients, carriers, stabilizers and other auxiliary substances is available in REMINGTONS PHARMACEUTICAL SCIENCES (Mack Pub. Co., N. J. 1991), incorporated herein by reference.

In order to augment an immune response in a subject, the compositions and methods described herein can further include ancillary substances/adjuvants as well as the compound of the invention, such as pharmacological agents, cytokines, or the like. Suitable adjuvants include any substance that enhances the immune response of the subject to the antigens attached to the microparticles of the invention. They may enhance the immune response by affecting any number of pathways, for example, by stabilizing the antigen/MHC complex, by causing more antigen/MHC complex to be present on the cell surface, by enhancing maturation of APCs, or by prolonging the life of APCs (e. g., inhibiting apoptosis).

Typically adjuvants are co-administered with the vaccine or microparticle. As used herein the term "adjuvant" refers to any material that enhances the action of a antigen or the like.

Thus, one example of an adjuvant is a "cytokine". As used herein, the term "cytokine" refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth, proliferation or maturation. Certain cytokines, for example TRANCE, flt-3L, and CD40L, enhance the immunostimulatory capacity of APCs. Non-

limiting examples of cytokines which may be used alone or in combination include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1 a), interleukin-11 (IL-11), MIP-la, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO), CD40 ligand (CD40L), tumor necrosis factor-related activation-induced cytokine (TRANCE) and flt3 ligand (flt-3L). Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Genentech (South San Francisco, CA), Amgen (Thousand Oaks, CA), R & D Systems and Immunex (Seattle, WA).

The sequence of many of these molecules are also available, for example, from the GenBank database. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (for example, recombinantly produced or mutants thereof) and nucleic acid encoding these molecules are intended to be used within the spirit and scope of the invention.

A composition which contains the microparticles of the invention and an adjuvant, or a vaccine or microparticles of the invention which is co-administered with an adjuvant, displays "enhanced immunogenicity" when it possesses a greater capacity to elicit an immune response than the immune response elicited by an equivalent amount of the vaccine administered without the adjuvant. Such enhanced immunogenicity can be determined by administering the adjuvant composition and microparticle controls to animals and comparing antibody titers and/or cellular-mediated immunity between the two using standard assays such as radioimmunoassay, ELISAs, CTL assays, and the like, well known in the art.

In the method of the invention the microparticles of the invention are typically delivered in liquid form or delivered in powdered form. Liquids containing the microparticles of the invention are typically suspensions. The microparticles of the invention may be administered in a liquid acceptable for delivery into an individual. Typically the liquid is a sterile buffer, for example sterile phosphate-buffered saline (PBS).

The microparticles of the invention are typically delivered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally or by infusion techniques. A physician will be able to determine the required route of administration for each particular patient.

The vaccine or microparticles are typically delivered transdermally. The term

"transdermal" delivery intends intradermal (for example, into the dermis or epidermis), transdermal (for example,"percutaneous") and transmucosal administration, for example, delivery by passage of an agent into or through skin or mucosal (for example buccal, conjunctival or gum) tissue. See, for example, Transdermal Drug Delivery: Developmental Issues and Research Initiatives, Hadgraft and Guy (eds.), Marcel Dekker, Inc., (1989); Controlled Drug Delivery : Fundamentals and Applications, Robinson and Lee (eds.), Marcel Dekker Inc., (1987); and Transdermal Delivery of Drugs, Vols. 1- 3, Kydonieus and Berner (eds.), CRC Press, (1987).

Delivery may be via conventional needle and syringe for the liquid suspensions containing microparticle particulate. In addition, various liquid jet injectors are known in the art and may be employed to administer the microparticles. Methods of determining the most effective means and dosages of administration are well known to those of skill in the art and will vary with the delivery vehicle, the composition of the therapy, the target cells, and the subject being treated. Single and multiple administrations can be carried out with the dose level and pattern being selected by the attending physician. The liquid compositions are administered to the subject to be treated in a manner compatible with the dosage formulation, and in an amount that will be prophylactically and/or therapeutically effective.

The microparticles themselves in particulate composition (for example, powder) can also be delivered transdermally to vertebrate tissue using a suitable transdermal particle delivery technique. Various particle delivery devices suitable for administering the substance of interest are known in the art, and will find use in the practice of the invention. A transdermal particle delivery system typically employs a needleless syringe to fire solid particles in controlled doses into and through intact skin and tissue. Various particle delivery devices suitable for particle-mediated delivery techniques are known in the art, and are all suited for use in the practice of the invention. Current device designs employ an explosive, electric or gaseous discharge to propel the coated core carrier particles toward target cells. The coated particles can themselves be releasably attached to a movable carrier sheet, or removably attached to a surface along which a gas stream passes, lifting the particles from the surface and accelerating them toward the target. See, for example, U.S. Patent No. 5,630,796 which describes a needleless syringe. Other needleless syringe configurations are known in the art.

Delivery of particles from such particle delivery devices is practiced with particles

having an approximate size generally ranging from 0.1 to 250μm. The actual distance which the delivered particles will penetrate a target surface depends upon particle size (e. g., the nominal particle diameter assuming a roughly spherical particle geometry), particle density, the initial velocity at which the particle impacts the surface, and the density and kinematic viscosity of the targeted skin tissue. In this regard, optimal particle densities for use in needleless injection generally range between about 0.1 and 25 g/cm3, preferably between about 0.9 and 1.5 g/cm3, and injection velocities generally range between about 100 and 3,000 m/sec, or greater. With appropriate gas pressure, particles having an average diameter of 10-70 um can be accelerated through the nozzle at velocities approaching the supersonic speeds of a driving gas flow.

The powdered compositions are administered to the subject to be treated in a manner compatible with the dosage formulation, and in an amount that will be prophylactically and/or therapeutically effective.

Microparticles comprising prophylactically or therapeutically effective amount of the antigen described herein can be delivered to any suitable target tissue via the above-described particle delivery devices. For example, the compositions can be delivered to muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland and connective tissues.

A "therapeutically effective amount" is defined very broadly as that amount needed to give the desired biologic or pharmacologic effect. This amount will vary with the relative activity of the antigen to be delivered and can be readily determined through clinical testing based on known activities of the antigen being delivered. The 'Physicians Desk Reference" and "Goodman and Gilman's The Pharmacological Basis of Therapeutics" are useful for the purpose of determined the amount needed in the case of known pharmaceutical agents. The amount of microparticles administered depends on the organism(for example animal species), antigen, route of administration, length of time of treatment and, in the case of animals, the weight, age and health of the animal. One skilled in the art is well aware of the dosages required to treat a particular animal with an antigen.

Commonly, the microparticles are administered in microgram amounts. The coated microparticles are administered to the subject to be treated in a manner compatible with the dosage formulation, and in an amount that will be effective to bring about a desired immune response. The amount of the microparticles to be delivered which, is 1μg to 5 mg,

more typically 1 to 50, μg of peptide, depends on the subject to be treated. The exact amount necessary will vary depending on the age and general condition of the individual being immunized and the particular nucleotide sequence or peptide selected, as well as other factors. An appropriate effective amount can be readily determined by one of skill in the art upon reading the instant specification.

Mixed populations of different types of microparticles can be combined into single dosage forms and can be co-administered. The same antigen can be incorporated into the different microparticle types that are combined in the final formulation or co-administered. Thus, multiphasic delivery of the same antigen can be achieved. Alternatively, different antigens may be adsorbed onto the different microparticle types combined in a formulation. For example, a formulation may comprise a negatively charged antigen adsorbed to positively charged microparticles and a positively charged antigen adsorbed to negatively charged microparticles. Different antigens may therefore be co-administered in a single dosage form.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Examples

Materials

Benzoyl peroxide (BPO), polyvinylalcohol (molar mass 49000), styrene, succinic anydride, methyl methacrylate were purchased from Aldrich. Methacrylic acid / ethylacrylate 1/1 (mol/mol) statistical copolymer (trade name Eudragit ® L100-55) was supplied by Röhm Pharma as a powder sample and is characterized by a number-average molar mass of 250000 g/mol. Butyl methacrylate/2-dimethylamino ethyl methacrylate/methyl methacrylate 1/2/1 (mol/mol) copolymer (trade name Eudragit ® E100) was supplied by Röhm Pharma as a powder sample and is characterized by a number-average molar mass of 150000 g/mol. BSA and Bradford Reagent were purchased from Sigma. Methanol (99,9%, Carlo Erba) and 2,2'-azobis (isobutyronitrile) (AIBN) (98.0%, Fluka) were used without further purification. Methyl methacrylate (MMA) (99%, Aldrich) was distilled under vacuum immediately before use.

Synthesis of fluorescent monomer

2.0 g of fluoresceine (6.0 mmol), 2.0 g of calcium carbonate and hydroquinone (trace) were dissolved in 100 ml of DMF, and the solution was heated at 60°C. Allyl chloride was added slowly dropwise and the reaction was allowed to proceed for 30 h in the dark. After vacuum evaporation of the solvent the product was purified by flash column chromatography (silica gel; diethyl ether-ethyl acetate 80:20 as eluent). Yield 53%, (m.p.=123-125°C); MS, m/z (%): 412 (M+, 100), 371 (10), 287 (20), 259 (15), 202 (7); ^{1}H-NMR (CD_3OD): δ 4.44 (dd, J=5.9 and 1 Hz, 2 H, O-CH_2-CH=), 4.75 (dd, J=5.9 and 1 Hz, 2 H, O-CH_2-CH=), 5.08 (m, 2H, CH_2=CH), 5.40 (m, 2H, CH_2=CH), 5.58 (m, 1H, CH_2=CH), 6.10 (m, 1H, CH_2=CH), 6.60 (m, 2H, Ar), 6.98 (m, 3H, Ar), 7.25 (d, J=1 Hz, 1H, Ar), 7.45 (dd, J=7.5 and 1 Hz, 1H, Ar), 7.85 (m, 2H, Ar), 8.30 (dd, J=7.5 and 1Hz, 1H, Ar).

Acid Microparticles

Microparticles A4 and A7 were prepared by dispersion polymerization of styrene (monomer) in the presence of hemisuccinated poly(vinyl alcohol) as the steric stabilizer. Microparticles 1D, 1E, H1D and fluorescent H1D were obtained by dispersion polymerization of methyl methacrylate (monomer) in the presence of Eudragit® L100-55 as the steric stabilizer. The microparticles were produced by dispersion polymerization. The physico-chemical properties of these mircoparticles are described in Table 1 below.

As a typical example, the preparation of the microparticle sample A7 (polystyrene and hemisuccinated polyvinylalcohol) was as follows: 1.86 g of hemisuccinated polivinylalcohol, 15.5 ml of styrene, 1.95 g of BPO were dissolved in 162 ml of ethanol/2-methoxyethanol 1/1 under a nitrogen atmosphere; three freeze-thaw cycles were run. A4 microparticles were prepared with a similar procedure starting from 1.34 g of hemisuccinated polyvinylalcohol dissolved in 162 ml of ethanol/2-methoxyethanol 9/1. The solution was heated at 78°C for 48 hours under mechanic stirring (60 rpm). The reaction mixture was then cooled and, after three cycles of centrifugation and redispersion with the organic solvent and two cycles with HPLC grade water, the resulting particles were lyophilized. The resulting yields were 76% and 82% respectively.

As a typical example for the Eudragit stabilized polymethylmethacrylate microparticles, the preparation of the sample 1D is described: 14.73 g of Eudragit was dissolved under a nitrogen atmosphere for 30 min in 200 ml methanol heated at 68°C. A

0.37 g portion of 2,2'-azobis (isobutyronitrile) (AIBN) was dissolved in 18.4 g of methylmethacrylate monomer and added to solution. 1E microparticles were prepared in a similar way starting from 18.10 g of Eudragit. The reaction was left to proceed for 24 hrs under constant stirring. The reaction mixture was then cooled and, after three cycles of centrifugation and redispersion with methanol and then two cycles with deionized water, the resulting particles were lyophilized. The resulting yields were 78% and 65% respectively.

As a further typical example for the Eudragit stabilized polymethylmethacrylate microparticles, the preparation of sample H1D is described: 7.36 g of Eudragit L 100-55 were dissolved in 200 ml of a solution of methanol/water 76/24 wt-% and heated at 60°C with mechanical stirring (speed of stirring 300 g/min) under nitrogen atmosphere and reflux condenser. After 30 min, 370 mg (2.25 mmol) of AIBN, dissolved in 18.3 g (183 mmol) of methyl methacrylate, were added to the solution and the reaction was allowed to proceed for 24h. At the end of the reaction, the latex was cooled, filtered and then purified by four cycles of centrifugation (2000 g/min for 10 minutes) and redispersion with methanol and deionized water. The reaction yield was 76.2%, as determined gravimetrically. Fluorescent H1D was obtained by reacting fluorescent monomer (see above) with together methyl methacrylate in the dispersion reaction. 11.0 g of Eudragit L 100-55 was dissolved in 200 ml of a solution of methanol water 76/24 wt-% and heated at 60°C with mechanical stirring (speed of stirring 300 g/min) under nitrogen atmosphere and reflux condenser. After 30 min, 370 mg (2.25 mmol) of AIBN and 5.0 mg (12.1 μmol) of fluorescent monomer, dissolved in 18.3 g (183 mmol) of methyl methacrylate, were added to the solution and the reaction was allowed to proceed for 24 h. At the end of the reaction, the microparticles were purified as previously described.

Basic Microparticles

Microparticles HE1D (diameter 0.48 μm ± 0.03) were prepared by dispersion polymerization of methyl methacrylate (monomer) in the presence of Eudragit® E100 as the steric stabilizer. 14.73 g of Eudragit were dissolved in 200ml of a solution of methanol/water 76/24 wt-% and heated at 60°C with mechanical stirring (speed stirring 300 g/min) under nitrogen atmosphere and reflux condenser. After 30 min, 370 mg (2.25 mmol) of AIBN dissolved in 18.3 g (183 mmol) of MMA were added to the solution and the reaction was allowed to proceed for 24 hr. At the end of the reaction the microparticles

were purified as previously described.

Physico-chemical characterization

i) Morphological characterization: particle size and size distribution were measured using a JEOL JSM-35CF scanning electron microscope (SEM) operating at an accelerating voltage of 20 kV. The samples were sputter coated under vacuum with a thin layer (10-30 Å) of gold. The SEM photographs were digitalizated and elaborated by the Scion Image processing program. From 200 to 250 individual microsphere diameters were measured for each sample.

ii) Determination of amount of steric stabilizer on the external surface of the microparticles: for acidic microparticles, the amount of steric stabilizer linked to the microparticle surface was determined by back titration of the excess NaOH after complete salification of the acid groups and microparticle removal by centrifugation. The salification was accomplished by dispersing in a beaker 0.6 g of a microparticle sample in 10 ml of 20 mM NaOH at room temperature for 24 h. Then, the microparticle sample was removed by centrifugation and washed twice with 25 ml of distilled water. The supernatants were combined and the excess NaOH was titrated with 20 mM HCl.

For basic microparticles, the amount of steric stabilizer was determined by back titration of the excess HCl after complete salification of the aminic groups and microparticles removal. The salification was accomplished by dispersing in a beaker 0.6 g of a microparticle sample in 10 ml of 20 mM HCl at room temperature. The microparticles were removed by centrifugation and washed twice with water. The supernatants were combined and the excess HCl was titrated with 20 mM NaOH.

The physico-chemical properties of the acidic microparticles are shown in Table 1 below.

Table 1. Acid microparticles physico-chemical characterization

Sample	SEM diameter (μm)	Surface charge density (μmol/g)	Surface charge density (μmol/m^2)
A4	0.99 ± 0.03	8.1	72.5
A7	3.46 ± 0.10	4.6	30.9
1D	4.35 ± 1.02	48.1	37.8
1E	2.60 ± 0.45	59.2	27.3
H1D	1.69 ± 0.16	62.1	17.8
H1Dfluo	2.13 ± 0.09	59.2	21.1

Protein adsorption

As a typical example, the adsorption behaviour of BSA and trypsin, as model proteins, was investigated on the H1D and HE1D. 5.0 mg of H1D or HE1D was incubated in 1.0 ml of a 20 mM sodium phosphate buffer solution at pH 7.4 in the presence of BSA or trypsin at different concentrations (from 10 to 250 μg/ml) for 2 h. Then, the microparticle sample was removed by centrifugation at 15000 g/min for 10 min and the amount of the residual BSA or trypsin on the supernatant was estimated using the Bradford colorimetric method (Bradford, M.M. Anal. Biochem. 1976, 72, 248). The amount of adsorbed BSA or trypsin was then calculated as the difference between the feed and the residual BSA or trypsin. The amount of adsorbed protein increased as the protein concentration increased which suggested a high compatibility of protein toward the microparticle surface (see Figure 1).

Tat polypeptide

The biologically active Tat protein of HIV-1 (HTLVIII-BH10) was produced in Escherichia coli, purified as a good laboratory practice (GLP) manufactured product and tested for activity as previously described (Ensoli B, Buonaguro L, Barillari G, *et al*, Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation, J. Virol., 1993; 67:277-87; Ensoli B,

Gendelman R, Markham P, *et al*, Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma, Nature, 1994; 371:674-80; Fanales-Belasio E, Moretti S, Nappi F, et al., Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses, J. Immunol., 2002; 168:197-206; and Chang HC, Samaniego F, Nair BC, Buonaguro L, Ensoli B., HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region, AIDS, 1997; 11:1421-31). To prevent oxidation that occurs easily because Tat contains seven cysteines, the Tat protein was stored lyophilized at –80°, and resuspended immediately before use in degassed sterile PBS (2 mg/ml) for adsorption to the microparticles, or in degassed PBS containing 0.1% bovine serum albumine (BSA) (Sigma, St. Louis, MI) for serological assays, as described (Fanales-Belasio *et al* supra). In addition, since Tat is photo- and thermosensitive, the handling of Tat was always performed in the dark and on ice. Endotoxin concentration of different GLP lots of Tat was always below the detection limit (<0.05 EU/mg), as tested by the Limulus Amoebocyte Lysate analysis.

Tat peptides

15 amino acid Tat-derived peptides (C-terminal amide) were synthesized using standard methods (Table 2). To predict Tat CTL epitopes for the K^d allele, the HLA peptide motif search (http://bimas.dcrt.nih.gov/molbio/hla_bind/) was used.

Table 2. *Tat-derived 15-mer peptides*

Peptide	Aminoacid position	Amino acid sequence[a]
TC27	1-15	MEPVDPRLEPWKHPG
TC28	6-20	PRLEPWKHPGSQPKT
TC29	11-25	WKHPGSQPKTACTNC
TC30	16-30	SQPKTACTNCYCKKC
TC31	21-35	ACTNCYCKKCCFHCQ
TC32	26-40	YCKKCCFHCQVCFIT
TC33	31-45	CFHCQVCFITKALGI
TC34	36-50	VCFITKALGISYGRK
TC35	41-55	KALGISYGRKKRRQR
TC36	46-60	SYGRKKRRQRRRPPQ
TC37	51-65	KRRQRRRPPQGSQTH
TC38	56-70	RRPPQGSQTHQVSLS
TC39	61-75	GSQTHQVSLSKQPTS
TC40	66-80	QVSLSKQPTSQSRGD
TC41	71-85	KQPTSQSRGDPTGPK
TC42	76-90	QSRGDPTGPKEQKKK

[a]Peptides were designed based on HIV-1 (BH10) Tat 102 aa long.

Absorption of Tat to the microparticles

Microparticles were resuspended (2 mg/ml) in degassed sterile phosphate buffered saline (PBS) and stored at 4°C prior to use.

To prepare Tat-microparticle complexes, the appropriate volume of Tat and microparticles were incubated in the dark and on ice for 60 min, and spun at 13,000 rpm for 10 min. The pellets (Tat-microparticle complexes) were resuspended in the appropriate volume of degassed sterile PBS and used immediately.

Flow cytometry

Microspheres (50 μg) were incubated with increasing amount of the Tat protein (0.1, 1, 2, 5 and 10 μg) in a final volume of 50 μl for 60 min at room temperature under mild agitation. Microspheres alone or microsphere-Tat complexes were spun at 13.000

rpm for 15 min, washed twice and resuspended in 50 µl of PBS. 5µl of microspheres-Tat complexes or microspheres alone were then incubated for 30 min at 4°C with a FITC-labeled anti-Tat monoclonal antibody (Intracel, Issaquah, WA), or with a FITC-labeled anti-Tat rabbit polyclonal antibody, prepared in house (Magnani et al., unpublished results) and analyzed by flow cytometry (FacScan Becton-Dickinson Mountain View, CA).

The results indicated that Tat adsorbs at the surface of the A4, A7, 1D, 1E and H1D microparticles (Figure 2). Although the maximum fluorescence was detected with 1 µg of Tat, the result was not quantitative and did not really represent the loading efficiency of the microparticles, but it was likely due to antibody steric hindrance, as indicated by the experiments described in the following sections. Both classes of microparticles (those obtained by dispersion polymerization of styrene (monomer) in the presence of hemisuccinated poly(vinyl alcohol) and those obtained by dispersion polymerization of methyl methacrylate (monomer) in the presence of Eudragit® L100-55) were stable and could be stored as a lyophilisate or suspension for several months. Changes in terms of their capacity to adsorb (and to release) Tat has been found in microparticles suspensions stored at + 4°C after more than 8 months.

Cell cultures

Monolayer cultures of human HL3T1 cells, containing an integrated copy of plasmid HIV-1-LTR-CAT, where expression of the chloramphenicol acetyl transferase (CAT) reporter gene is driven by the HIV-1 LTR promoter, were obtained through the NIH AIDS research and reference reagents program (Bethesda, MD) and grown in DMEM (Gibco, Grand Island, NY) containing 10% FBS (Gibco).

Isolation of murine and human primary cells

Six-weeks old Swiss female mice (Nossan, Italy) were injected intraperitoneally (i.p.) with 1.0 ml of 10% thioglycolate (Sigma). At 4 days, mice were sacrificed, and peritoneal exudate cells highly enriched for macrophages were harvested by i.p. lavage with 10 ml of ice-cold Hank's balanced salt solution supplemented with 10 U/ml of heparin. Cells ($4x10^6$ cells) were washed twice, resuspended in DMEM supplemented with 10% heat-inactivated FBS, 1% antibiotics, 2 mM glutamine, seeded onto 35 mm Petri dishes, and incubated for 12 h in a humidified 5% CO_2 atmosphere at 37°C to allow macrophage adherence. Nonadherent cells were gently removed with warmed DMEM

medium. Monolayers were 95% pure macrophages as determined by immunostaining and surface marker analysis using a rat monoclonal antibody to mouse F4/80 (Caltag Lab., Burlingame, CA).

Murine splenocytes were purified from spleens of 10-weeks old Balb/c female mice using Ficoll gradients (Caselli E et al., J. Immunol., 1999;162:5631-8) and grown in RPMI 1640 supplemented with 10% FBS. Human monocytes and monocyte-derived dendritic cells were purified from a buffy coat, characterized and cultured as described (Micheletti F et al., Immunol., 2002;106:395-403).

Analysis of cytotoxicity in vitro

HL3T1 cells (1 x 10^4/100 µl) were seeded in 96-well plates and cultured at 37°C for 24 h. One-hundred µl of medium containing the microspheres alone (10, 30, 50, 100, 300, 500 and 1000 µg/ml) or bound to Tat (1 µg/ml) (sextupled wells) were then added to the cells. Untreated cells and cells incubated with Tat alone were the controls. Cells were incubated for 96 h at 37°, and cell proliferation was measured using the colorimetric cell proliferation kit I (MTT based) provided by Roche (Roche, Milan, Italy) (Mosmann T., J. Immunol. Meth., 1983;65:55-63).

Both classes of microparticles (those obtained by dispersion polymerization of styrene (monomer) in the presence of hemisuccinated poly(vinyl alcohol) and those obtained by dispersion polymerization of methyl methacrylate (monomer) in the presence of Eudragit® L100-55) and microparticle-Tat complexes were not toxic to the cells up to 50 µg/ml as compared to untreated or Tat-treated cells ($p<0.01$) (Figure 3). A 50% reduction of cell viability was observed only at higher doses (300-1000 µg/ml) (data not shown).

Cellular uptake of fluorescent H1D microspheres

HL3T1 cells (1 x 10^5) were seeded in 24-well plates containing a 12-mm glass coverslip, and incubated with fluoresceinated-H1D microspheres. After incubation, cells were washed, fixed with 4% cold paraformaldehyde and observed at a confocal laser scanning microscope LSM410 (Zeiss, Oberkochen, Germany). Image acquisition, recording and filtering were carried out using a Indy 4400 graphic workstation (Silicon Graphics, Mountain View, CA) as previously described (Neri LM et al., Microsc. Res. Tech., 1997;36:179-87).

Human monocytes and monocyte-derived dendritic cells (1 x 10^5), and murine splenocytes (4 x 10^6) were incubated in 24-well plates with fluorescent-H1D microparticles for 24 h. After incubation, cells were washed and layered onto glass slides previously coated with poly-L-lysin (Sigma) according to manufacturer's instructions. Cells were fixed with 4% cold paraformaldehyde, stained with DAPI (Sigma) and observed with a confocal microscope, as described above, and at a fluorescent microscope Axiophot 100 (Zeiss). The green fluorescence (microparticles) was observed with a 450-490 λ, flow through 510 λ and long pass 520 λ filter; the blue fluorescence (DAPI) was observed with a band pass 365 λ, flow through 395 λ and long pass 397 λ filter. For the same microscopic field, green, blue and phase contrast images were taken with a Cool-Snapp CCD camera (RS-Photometrics, Fairfax, VA). The three images were then overlapped using the Adobe Photoshop 5.5 program.

Murine macrophages (3 x 10^6) were incubated in the presence of microparticles, at a ratio of 4 microparticles per macrophage, for 1, 2 and 4 h. Cells were extensively washed to remove non-phagocytosed microparticles, fixed with 2% paraformaldehyde and 2.5% glutaraldehyde for 30 min at 4°C, and stained with toluidine blue. Cells were observed at a phase contrast microscope (100X) to count the number of macrophages with phagocytosed microparticles.

All particles were taken up by murine macrophages with similar kinetics and percentage of phagocytosis (Figure 4). Similar results were obtained when human monocytes, monocyte-derived dendritic cells, murine splenocytes and HL3T1 cells, were cultured with fluorescent-H1D microparticles and observed with confocal and fluorescent microscopy (Figure 5). This data indicated that the microparticles are taken up by different cell types and that chemical composition and size do not affect their phagocytosis.

Immunofluorescence

HL3T1 cells (1 x 10^5) were seeded in 24-well plates containing a 12-mm glass coverslip, and incubated with fluoresceinated-H1D microparticles-Tat protein complexes. The dose of 30 μg/ml of miscrospheres associated with 5 μg/ml of Tat was used. Controls were represented by cells incubated with the Tat (5 μg/ml) protein alone or untreated cells. After incubation, cells were washed, fixed with 4% cold paraformaldehyde and analyzed by immunofluorescence with an anti-Tat monoclonal antibody (4B4C4) and a goat Cy3-conjugated anti-mouse IgG secondary serum, as previously described (Betti M et al.,

Vaccine, 2001;19:3408-19). Cells were colored with DAPI and observed at a fluorescence microscope. The red fluorescence (Tat) was observed with a band pass 546 λ, flow through 580 λ and long pass 590 λ filter; the green (microparticles) and blue fluorescence (DAPI) were observed as described above. For the same microscopic field, green, red, blue and phase contrast images were taken and overlapped as described above.

The Tat-microparticle complexes were readily taken up by the cells and the Tat protein was released intracellularly in the proximity of the nucleus (Figure 6). Tat was released in a controlled fashion, as suggested by the observation that after 48 h Tat-loaded particles were still detectable in the cells (Figure 5).

Evaluation of the Tat protein activity

HL3T1 cells (5×10^5) were seeded in 60-mm Petri dishes. 24 h later cells were replaced with 1 ml of fresh medium and incubated with Tat alone (0.1, 0.25, 0.5, 1 μg/ml) or Tat bound to the microparticles (30 μg/ml) in the absence or presence of 100 μM chloroquine (Sigma). In some experiments, Tat alone or Tat-microparticle complexes were exposed to air and light at room temperature for 16 h before the addition to the cells. CAT activity was measured 48 h later in cell extracts after normalization to total protein content, as described previously (Betti M et al., Vaccine, 2001;19:3408-19).

Exposure to air and light did not inactivate Tat trans-activating function when Tat was previously adsorbed onto the microparticles, whereas it caused the loss of Tat biological activity when Tat was free (Figure 7). Thus, Tat bound to the microparticles was protected from oxidation.

Expression of CAT was maximal and similar among all Tat-microparticle complexes (Figure 8). In addition, at the doses of 100, 250 and 500 ng/ml of Tat bound to the microparticles, CAT expression was significantly higher than that elicited by the same doses of Tat alone (Figure 8), suggesting that Tat bound at the surface of the microparticles is protected from proteolytic degradation and/or released in a controlled fashion from the complexes, in agreement with the previous results shown earlier (Figure 7). These results demonstrate that all the microparticles tested adsorb and release biologically active Tat protein in a dose dependent fashion, and that Tat bound to the microparticles maintains its native conformation and biological activity.

Gel electrophoresis

Microspheres (50 μg) were incubated with increasing amounts of the Tat protein in a final volume of 50 μl for 60 min at room temperature under mild agitation. Microsphere-Tat complexes were spun at 13.000 rpm for 15 min, washed twice in PBS, and resuspended in 30 μl of NaCl 0.9%, phosphate buffer 5 mM. Samples were boiled for 5 min and spun at 13.000 for 15 min. Supernatants were run onto 14% SDS-polyacrylamide gels and colored with Coomassie blue (Davis LG, Dibner MD, Battey JF. In: Davis LG, Dibner MD, Battey JF, editors. Basic Methods in Molecular Biology. New York: Elsevier, 1986.).

Exposure of free Tat to oxidizing conditions caused the disappearance of the monomeric bioactive form of Tat and, concomitantly, the appearance of oxidized Tat multimers, as compared to free Tat not exposed to air and light (data not shown). In contrast, when Tat was bound to the microspheres, the monomeric conformation of Tat was the most abundant form, either before or after exposure to air and light (data not shown). This result demonstrated that adsorption to the microspheres preserves Tat native conformation and protects it from oxidation, in agreement with the functional Tat trans-activation assay, shown earlier (Fig. 6).

Mice immunization

Animal use has complied with national guidelines and institutional policies. Seven-eight-weeks-old female Balb/c mice (H-2^d) (Nossan, Milan, Italy) were immunized with 0.5 μg of Tat protein adsorbed to 30 μg of microparticles, Tat protein alone or Tat protein and Freund's adjuvant (CFA for the first immunization, IFA for subsequent immunizations). Control mice were injected with PBS alone. Immunogens (100 μl) were given by intramuscular (i.m.) injections in the quadriceps muscles of the posterior legs. Four separate experiments were performed. Mice were immunized at weeks 0 and 2 (2 experiments), and at weeks 0 and 4 (2 experiments). Animals were controlled twice a week at the site of injection, for the presence of edema, induration, redness, and for their general conditions, such as liveliness, vitality, weight, motility, sheen of hair. No signs of local nor systemic adverse reactions were ever observed in mice receiving the Tat-microparticle complexes as compared to mice vaccinated with Tat alone or to untreated mice. Only mice inoculated with Freund's adjuvant developed a visible granuloma at the site of injection. The immune response was evaluated two weeks after immunization. At sacrifice mice were anesthetized intraperitoneally with 100 μl of isotonic solution

containing 1 mg of Inoketan (Virbac, Milan, Italy), and 200 mg Rompun (Bayer, Milan, Italy).

Anti-Tat serology

To determine whether the chemical composition and the size of the microparticles influence the type and the strength of the immune response to HIV-1 Tat, mice (n=10) were immunized i.m. with 0.5 μg of Tat protein adsorbed to 30 μg of polystyrene (A4 and A7), and polymethyl methacrylate (1D, 1E and H1D) microparticles. In addition, three groups of mice were immunized with Tat alone (n=6), Tat and Freund's adjuvant (n=10) or PBS (n=10). Two weeks after the first immunization, half number of mice by treatment group was sacrificed. At the same time, the remaining mice received the second immunization and they were sacrificed two weeks later.

Serological responses of individual mice were measured by enzyme-linked immunosorbent assay (ELISA) in 96-wells immunoplates (Nunc Immunoplate F96 Polysorp, Nunc, Naperville, IL). Wells were coated with 100 μl of Tat protein (1 μg/ml in 0.05 M carbonate buffer pH 9.6). Plates were sealed and incubated in the dark for 12 hours at 4°C. After extensive washes with 0.05% Tween 20 in PBS (PBS-Tween) in an automated washer (Immunowash 1575, Bio-Rad Laboratories, Hercules, CA), plates were blocked with 150 μl/well of PBS containing 3% BSA for 120 min at 37°C, washed and then incubated with 100 μl/well of the mice sera in duplicate wells, diluted from 1:195 up to 1:100.000, for 90 min at 37°C, and washed extensively. Immunocomplexes were detected with 100 μl/well of a horse-radish peroxidase (HRP) conjugated sheep anti-mouse IgG (Amersham Life Science, Little Chalfont, Buckinghamshire, England), diluted 1:1000 in PBS-Tween containing 1% BSA. Plates were incubated for 90 min at room temperature, washed 5 times and incubated with 100 μl/well of peroxidase substrate (ABTS) (Roche, Milan, Italy) for 40 min at room temperature. The reaction was blocked with 100 μl of 0.1 M citric acid and the absorbance was measured at 405 nm in an automated plate reader (ELX-800, Bio-Tek Instruments, Winooski, UT). The cutoff corresponded to the mean OD_{405} (+ 3 SD) of sera of control mice inoculated with PBS, tested in three independent assays. For anti-Tat IgG epitope mapping, eight synthetic peptides (aa 1-20, 21-40, 36-50, 46-60, 56-70, 52-72, 65-80, 73-86) representing different regions of Tat (HTLVIII-BH10) were diluted in 0.1 M carbonate buffer (pH 9.6) at 10 μg/ml, and 96-well immunoplates were coated with 100 μl/well. The assays were

performed as described above. The cutoff for each peptide corresponded to the mean OD_{405} (+ 3 SD) of sera of control mice injected with PBS, tested in three independent assays.

For anti-Tat IgG isotyping, plates were coated with Tat protein and incubated with mice sera diluted 1:100 and 1:200, as described above. After washing, 100 µl of goat anti-mouse IgG1, or IgG2a (Sigma), diluted 1:100 in PBS-Tween containing 1% BSA, were added to each well. Immunocomplexes were detected with a horse-radish peroxidase-labeled rabbit anti-goat IgG (Sigma) diluted 1:7500 in PBS-Tween containing 1% BSA, as described above. The cutoff for each IgG subclass corresponded to the mean OD_{405} (+ 3 SD) of sera of control mice injected with PBS, tested in three independent assays.

Serum antibody responses were monitored by ELISA at sacrifice. All five groups of mice immunized with the Tat/microparticle complexes developed specific anti-Tat antibodies, that were detectable after the second immunization and with titers similar among the five treatment groups and to Tat-vaccinated mice (Table 3).

Table 3. *Humoral immune response to Tat protein after immunization with Tat/microparticle complexes*[a]

Group	I Immunization	II Immunization
A4/Tat	0/5 (0)	5/5 (2109 ± 2611)
A7/Tat	0/5 (0)	3/5 (624 ± 652)
1D/Tat	0/5 (0)	5/5 (4687 ± 2210)
1E/Tat	0/5 (0)	5/5 (1093 ± 1270)
H1D/Tat	0/5 (0)	5/5 (6874 ± 10.385)
Tat	2/3 (130 ± 112)	3/3 (9635 ± 13.358)

[a] Mice were immunized once (I immunization) or twice (II immunization), at weeks 0 and 2, and sacrificed two weeks later. The antibody response was determined on serially diluted sera of individual mice by ELISA using Tat protein as the antigen. Results of one representative experiment are expressed as the number of responder mice vs the total number of immunized mice. In each group the mean titers ± SD of the responders are reported in parenthesis. The differences in Ab titers of mice immunized with the Tat/microparticle complexes as compared to mice vaccinated with Tat alone were not significant ($p > 0.01$).

The epitope reactivity of the antibodies was directed to the NH_2-terminal region of the protein (residues 1-20) in all mice of all treatment groups immunized with the Tat/microparticle complexes, or Tat. A second reactive epitope was identified at residues 21-40 only in the serum of two mice, one immunized with A4/Tat (mouse ID 10) and the other immunized with 1D/Tat (mouse ID 9) (data not shown).

The isotype analysis of the IgG subclasses indicated the presence of both IgG1 and IgG2a isotypes. However, a prevalence of the IgG1 subclass was observed in all groups.

Purification of mouse splenocytes

Mononuclear cells were purified from spleens using cells strainers provided by Falcon. Cells were resuspended in PBS containing 20 mM EDTA, treated with a red blood cells lysis buffer (100 mM NH_4Cl, 10 mM $KHCO_3$, 10 μM EDTA) for 4 minutes at room temperature, and washed twice with RPMI 1640 (Gibco) without serum. Cells were resuspended in RPMI 1640 supplemented with 10 % heat-inactivated FBS (Hyclone), and counted by trypan blue exclusion dye. Purified splenocytes were pooled by treatment group, and used to evaluate the cellular immune responses.

Tat-specific T cell activation

Tat-specific T-cell activation was determined using different assays. 1) Splenocytes were cultured at 2 x 10^5/well (sextupled wells) in 200 μl of RPMI 1640 supplemented with 10% heat inactivated FBS in the presence of Tat protein (0.1, 1 or 5 μg/ml) or Con A (10 μg/ml) (Sigma) for five days. Methyl-^{3}H-thymidine (2.0 Ci/mmol; ICN) was added to each well (1 μCi) and cells were incubated for 16 h. [^{3}H]-Thymidine incorporation was measured with a β-counter. The S.I. was calculated by dividing the mean cpm of six wells of antigen-stimulated cells by the mean cpm of six wells of the same cells grown in the absence of the antigen. Values higher than the cutoff [mean S.I. (+ 2 SD) of the control mice injected with PBS alone] were considered positive.

2) Stable clones of murine Balb/c 3T3-Tat expressing cells and Balb/c 3T3-pRPneo-c (referred to as BALB/c-control cells) (H^{2d} haplotype) were grown in Dulbecco's minimal essential medium plus 10% FBS and G418 (350 μg/ml, Sigma). Mice splenocytes were co-cultivated at 20:1 ratio with BALB/c 3T3-Tat expressing cells in the presence of Tat (0.5 μg/ml). After 4 days of culture, rIL-2 (10 U/ml; Roche, Milan, Italy) was added to the cultures and cells grown for additional 48 hrs. γINF production was measured by ELISA on culture supernatants before and after addition of IL-2. Ninety-six wells immunoplates (Nunc Immunoplate F96 Polysorp) were coated with 100 μl of an anti-mouse γINF mAb (1 μg/ml; Endogen, Woburn, MA) in 0.03 M carbonate buffer for 16 h at 4°C. Empty wells were then blocked with 200 μl of PBS-4% BSA (assay buffer) for 1 h at room temperature, extensively washed with PBS-0.05% Tween 20 (washing buffer), and incubated with 50 μl of serially diluted cell supernatants for 1 h at room temperature. A titration curve (from 0 up to 20.000 pg/ml of recombinant murine γINF,

Euroclone, Devon, U.K.) was included in each plate. Each sample was tested in duplicate. Empty plates were then incubated with 50 µl/well of a biotine-labelled anti-mouse γINF mAb (400 ng/ml in assay buffer; Endogen) for 1 h at room temperature, extensively washed and incubated with HRP-labelled streptavidin (Endogen) diluted 1:6000 in assay buffer for 30 min at room temperature. Plates were washed, incubated with 100 µl/well of 3,3',5,5'-tetramethyl-benzidine (TMP; Sigma) substrate for 3 min, blocked with 100 µl/well 3 N HCl and the adsorbency read at 450 nm.

3) To measure the T-cell proliferation in response to Tat-derived 15-mer peptides, containing the computer predicted CTL epitopes for K^d allele, irradiated spleen cells (5 x 10^5) from naïve syngeneic Balb/c mice (serving as APC) were incubated in 96-flat bottom wells with 2 x 10^5 M of each Tat peptide for 1 hour. Splenocytes (1 x 10^5) from immunized mice, previously co-cultivated for 4 days with BALB/c 3T3-Tat expressing cells (at 20:1 ratio) in the presence of Tat (0.5 µg/ml) and purified using Ficoll gradients, were added to the wells in a final volume of 200 µl and final peptide concentration of 10^{-5} M. After 24 hours, aliquots of culture media were collected to measure the release of γIFN, whereas after additional 72 hours of culture cells were pulsed with methyl-^{3}H-thymidine (1 µCi/well) for 24 hours. Incorporated radioactivity was measured by liquid scintillation spectroscopy.

CD4+ T-cell proliferation in response to Tat was evaluated using mice splenocytes. Splenocytes of mice, obtained two weeks after the first or the second immunization, were cultured five days with 0.1, 1 and 5 mg/ml of Tat protein. Antigen-stimulated T-cell proliferation was determined by [^{3}H]thymidine incorporation (Table 4). After one immunization, specific responses to the highest dose of Tat were observed in splenocytes of all groups immunized with the Tat/microparticle complexes, and Tat. In addition, for the A7/ and 1E/Tat treatment groups Tat-specific CD4+ T-cell responses were detected also at the lower dose of 1 µg/ml of Tat. After two immunizations, Tat-specific T-cell proliferation was detected at both 1 and 5 µg/ml of Tat in all groups with and without the microparticles, and in addition, mice immunized with A4/Tat and 1D/Tat responded to as little as 0.1 µg/ml of recombinant Tat.

Table 4. *Lymphoproliferative response to Tat protein after immunization with Tat/microparticle complexes*[a]

	I Immunization				II Immunization			
Group	Tat 0.1 µg/ml	Tat 1 µg/ml	Tat 5 µg/ml	ConA 2 µg/ml	Tat 0.1 µg/ml	Tat 1 µg/ml	Tat 5 µg/ml	ConA 2 µg/ml
A4/Tat 0.5	1.08	2.66	12.71	21.14	2.02	13.02[b]	15.62	19.52
A7/Tat 0.5	1.51	3.21	19.05[b]	33.43	0.7	1.79	9.24	27.21
1D/Tat 0.5	0.81	1.73	14.49	67.38	6.60	15.66[b]	25.71	31.71
1E/Tat 0.5	1.30	4.17	13.37[b]	15.34	1.64	4.9	11.59	16.65
H1D/Tat 0.5	1.55	2.44	31.95[b]	38.97	1.7	3.51	14.25	20.11
Tat	n.d.	2.86	6.2[b]	75.8	n.d.	4.3[b]	27.03	40.6
PBS	1.67	2.66	4.04	5.3	1.84	1.46	5.6	18

[a] Mice were immunized at weeks 0 and 2, and immune response tested two weeks after the first and the second immunization. Cells were stimulated with recombinant Tat protein or ConA. Values represent the SI of murine splenocytes (pool of 5 spleens) after Tat or ConA activation. A SI higher than that of the control group injected with PBS was considered positive. [b]The differences in proliferative responses vs mice immunized with Tat alone were significant ($p < 0.05$).

In separate experiments, mice were immunized twice (at week 0 and 4) with the Tat/microparticle complexes. Splenocytes of mice, obtained two weeks after the second immunization, were co-cultured with BALB/c 3T3-Tat expressing cells in the presence of Tat. After 4 days of culture, the production of γINF in culture media of restimulated spleen cells was measured by ELISA. As shown in Figure 9, γINF production resulted

significantly increased in all five groups immunized with the Tat/microparticle complexes, as compared to mice injected with PBS. This effect comparable between the PS particles (A4 and A7) and, among the PMMA particles, it was greatly evident in the H1D/Tat treatment group. Thus, we measured the T-cell proliferation in response to Tat-derived peptides in two treatment groups, one for each type of microparticles. Splenocytes of mice vaccinate A4/Tat and H1D/Tat vaccinated mice, after co-cultivation with BALB/c 3T3-Tat expressing cells in the presence of Tat for 4 days, were purified and co-cultured with irradiated naive splenocytes pulsed with several Tat peptides. T cell proliferation was measured by ^{3}[H]thymidine incorporation after 96 hrs of culture, and γINF release was tested on aliquots of culture supernatants collected after 24 hrs of culture. The results of these experiments showed specific cell proliferation and release of γINF in response to TC34, TC38 and TC39 Tat peptides, containing computer predicted CTL epitopes for the K^{d} allele, in a fashion similar to Tat treated mice (Figure 10). In addition, although weaker, responses to other Tat peptides, including TC30, TC32, and TC41, were observed (Figure 10). Responses to other Tat-peptides were not observed (not shown).

Evaluation of the safety of Tat-microparticle complexes in vivo

At sacrifice animals were subjected to autopsy. Samples of cutis, subcutis and skeletal muscles at the sites of injection and other organs (lungs, heart, intestine, kidneys, spleen and liver) were fixed in 10% formalin for 12-24 h, embedded in paraffin, and routinely processed for histological examination. Three-5 μm paraffin-embedded sections were stained with hematoxylin and eosin, subjected to periodic acid-Shiff (PAS) reaction with and without diastase treatment (Sigma). Serial tissue sections were immuno-stained using the avidin-biotin-peroxidase complex technique (Vectastain ABC Kit PK-4002, Vector Labs, Burlingame, CA) according to Hsu et al. (J. Histochem. Cytochem. 1981;29:577-80). The panel of antibodies included S-100 (Dako, Denmark), HH-F 35 (Dako) for detection of α-actin, CD68 and Mac387 (Dako) for detection of macrophages. Briefly, after deparaffinization and rehydration, endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol; samples were then incubated with primary antibodies for 10-12 h at 4°C. Biotinilated-anti-mouse and anti-rabbit immunoglobulins (Sigma) were utilized as secondary antibodies. Specific reactions were detected following incubation with avidin-biotin-peroxidase conjugated and treatment with diaminobenzidine (Sigma) and hydrogen peroxide.

Histologically two types of pictures were observed at the site of injection. The first consisted of small foci, involving one or two muscle fibers, showing increased number of nuclei, and scarce macrophage infiltrate in the interstitial space (Figure 11A and C). These features were prevalently detected in mice injected with the Tat-microparticle complexes or Tat alone. The second type of picture was found in the muscular fascia and in the surrounding adipose tissue, and it was characterized by a central area of necrosis surrounded by neutrophil granulocytes and macrophages (Figure 11B and D). The macrophages always showed good reactivity to CD68 and Mac387 monoclonal antibodies; T and B lymphocytes were not detected in the inflammatory reactions. This type of lesion, as well as the higher number of inflammatory cells, was detected in the majority of mice receiving Tat and Freund's adjuvant. In the other animals and in control mice inoculated with PBS, the inflammatory reaction was inconspicuous, related to the traumatic stimulus or absent (data not shown). Laden macrophages reaction or other type of inflammatory reactions were not observed in the other organs.

No differences in the inflammatory reactions, related to the chemical composition and size of microparticles or the dose of Tat, were detected after one immunization. Indeed, only 2/22 (9%) mice, inoculated with A4-Tat 0.5 μg or 1D-Tat 0.5 μg, showed an inflammatory reaction. After two immunizations, 14/47 (30%) mice treated with the microparticle-Tat complexes developed a local inflammatory reaction. After three immunizations, 23/38 (60%) of mice treated with the Tat-microparticle complexes showed variable inflammatory reactions at the site of inoculation. In conclusion, the frequency of the inflammatory reactions correlated with the number of immunizations.

Tat-treated mice presented local inflammation (type one picture) only after the second inoculation in about 50% of the mice; macrophages infiltration was more frequently observed, but it was not related to the dose of Tat.

All mice treated with Tat and Freund's adjuvant showed intense inflammatory reactions independently from the number of immunizations; the incidence was more than 70% after the first injection and raised up to 90-100% after the second and the third treatment. This is likely due to the type of adjuvant used.

SEQUENCE LISTING

<110> INSTITUTO SUPERIORE DI SANITA

<120> USE OF MICROPARTICLES FOR ANTIGEN DELIVERY

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<160> 48

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            20                  25                  30

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        35                  40                  45

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    50                  55                  60

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His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
65                  70                  75                  80

ccg aca ggc ccg aag gaa cag aag aag aag gtg gag aga gag aca gag     288
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Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
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His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
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1               5                  10                  15

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Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
            20                  25                  30

cat tgc caa gtt tgt ttc ata aca aaa gcc tta ggc atc tcc tac ggc      144
His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly
        35                  40                  45

agg aag aag cgg aga cag cgt cga aga cct cct caa ggc agt cag act      192
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
    50                  55                  60

cat caa gtt tct cta tca aag caa ccc acc tcc caa tcc cga ggg gac      240
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His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly
        35                  40                  45

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65                  70                  75                  80

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cag cct aaa act gct ggt acc aat tgc tat tgt aaa aag tgt tgc ttt      96
Gln Pro Lys Thr Ala Gly Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
            20                  25                  30

cat tgc caa gtt tgt ttc ata aca aaa gcc tta ggc atc tcc tat ggc     144
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agg aag aag cgg aga cag cga cga aga cct cct caa ggc agt cag act     192
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cat caa gtt tct cta tca aag cag ccc acc tcc caa tcc cga ggg gac-    240
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20 25 30

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cag cct aaa act gct tgt acc aat tgc tat tgt aaa aag tgt tgc ttt      96
Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
            20                  25                  30

cat tgc caa gtt tgt ttc ata aca aaa gcc tta ggc atc tcc tat ggc     144
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agg aag aag cgg aga cag cga cga aga cct cct caa ggc agt cag act     192
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20 25 30

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35 40 45

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50 55 60

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His Cys Gln Val Cys Phe Ile Thr Ala Ala Leu Gly Ile Ser Tyr Gly
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20 25 30

cat tgc caa gtt tgc ttt ctg aac aaa ggc tta ggc atc tcc tat ggc 144
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35 40 45

agg aag aag cgg aga cag cga cga gga act cct cag agc agt aag gat 192
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65 70 75 80

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35 40 45

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50 55 60

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Thr Asp Arg Phe Asp
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20 25 30

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         35                  40                  45

agg aag aag cgg aga cag cga cga aga gct cct caa gac agt cag act      192
Arg Lys Lys Arg Arg Gln Arg Arg Arg Ala Pro Gln Asp Ser Gln Thr
    50                  55                  60

cat caa gtt tct cta tca aag caa ccc gcc tcc cag ccc cga ggg gac      240
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65                  70                  75                  80

ccg aca ggc ccg aag gaa tcg aag aag aag gtg gag aga gag aca gag      288
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Arg Lys Lys Arg Arg Gln Arg Arg Arg Ala Pro Gln Asp Ser Gln Thr
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His Gln Val Ser Leu Ser Lys Gln Pro Ala Ser Gln Pro Arg Gly Asp
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cag cct aaa act gct tgt aat aag tgt tat tgt aaa cac tgt agc tat      96
Gln Pro Lys Thr Ala Cys Asn Lys Cys Tyr Cys Lys His Cys Ser Tyr
            20                  25                  30

cat tgt cta gtt tgc ttt cag aca aaa ggc tta ggc att tcc tat ggc     144
His Cys Leu Val Cys Phe Gln Thr Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

agg aag aag cgg aga cag cga cga agc gct cct cca agc agt gag gat     192
Arg Lys Lys Arg Arg Gln Arg Arg Ser Ala Pro Pro Ser Ser Glu Asp
    50                  55                  60

cat caa aat ctt ata tca aag caa ccc tta ccc caa acc caa ggg gac     240
His Gln Asn Leu Ile Ser Lys Gln Pro Leu Pro Gln Thr Gln Gly Asp
65                  70                  75                  80

ccg aca ggc tcg gaa gaa tcg aag aag aag gtg gag agc aag aca gag     288
Pro Thr Gly Ser Glu Glu Ser Lys Lys Lys Val Glu Ser Lys Thr Glu
                85                  90                  95

aca gat cca ttc gat tag                                            306
Thr Asp Pro Phe Asp
            100
```

<210> 18
<211> 101
<212> PRT
<213> Human immunodeficiency virus

<400> 18

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1               5                  10                  15

Gln Pro Lys Thr Ala Cys Asn Lys Cys Tyr Cys Lys His Cys Ser Tyr
            20                  25                  30

His Cys Leu Val Cys Phe Gln Thr Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

Arg Lys Lys Arg Arg Gln Arg Arg Ser Ala Pro Pro Ser Ser Glu Asp
    50                  55                  60

His Gln Asn Leu Ile Ser Lys Gln Pro Leu Pro Gln Thr Gln Gly Asp
65                  70                  75                  80

Pro Thr Gly Ser Glu Glu Ser Lys Lys Lys Val Glu Ser Lys Thr Glu
                85                  90                  95

Thr Asp Pro Phe Asp
            100
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<210> 19
<211> 261

<212> DNA
<213> Human immunodeficiency virus

<220>
<221> CDS
<222> (1)..(261)

<400> 19

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1               5                   10                  15

cag cct agg act cct tgt aac aag tgt tat tgt aaa aag tgt tgc tat       96
Gln Pro Arg Thr Pro Cys Asn Lys Cys Tyr Cys Lys Lys Cys Cys Tyr
            20                  25                  30

cat tgc caa gtt tgc ttc ata acg aaa ggc tta ggc atc tcc tat ggc      144
His Cys Gln Val Cys Phe Ile Thr Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

agg aag aag cgg aga cag cga cga aga cct cct caa ggc ggt cag gct      192
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Gly Gln Ala
    50                  55                  60

cat caa gat cct ata cca aag caa ccc tcc tcc cag ccc cga ggg gac      240
His Gln Asp Pro Ile Pro Lys Gln Pro Ser Ser Gln Pro Arg Gly Asp
65                  70                  75                  80

ccg aca ggc ccg aag gaa tag                                         261
Pro Thr Gly Pro Lys Glu
                85
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<210> 20
<211> 86
<212> PRT
<213> Human immunodeficiency virus

<400> 20

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1               5                   10                  15

Gln Pro Arg Thr Pro Cys Asn Lys Cys Tyr Cys Lys Lys Cys Cys Tyr
            20                  25                  30

His Cys Gln Val Cys Phe Ile Thr Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Gly Gln Ala
    50                  55                  60

His Gln Asp Pro Ile Pro Lys Gln Pro Ser Ser Gln Pro Arg Gly Asp
65                  70                  75                  80

Pro Thr Gly Pro Lys Glu
                85
```

<210> 21
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<213> Human immunodeficiency virus

<220>
<221> CDS
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1 5 10 15

cag cct aca act cct tgt acc aaa tgc tat tgt aaa agg tgt tgc ttt 96
Gln Pro Thr Thr Pro Cys Thr Lys Cys Tyr Cys Lys Arg Cys Cys Phe
20 25 30

cat tgc caa tgg tgc ttt aca acg aag ggc tta ggc atc tcc tat ggc 144
His Cys Gln Trp Cys Phe Thr Thr Lys Gly Leu Gly Ile Ser Tyr Gly
35 40 45

agg aag aag cgg aga cag cga cga aga act cct caa agc agt cag ata 192
Arg Lys Lys Arg Arg Gln Arg Arg Arg Thr Pro Gln Ser Ser Gln Ile
50 55 60

cat caa gat cct gta cca aag caa ccc tta tcc caa gcc cga ggg aac 240
His Gln Asp Pro Val Pro Lys Gln Pro Leu Ser Gln Ala Arg Gly Asn
65 70 75 80

ccg aca ggc ccg aag gaa tcg aag aag gag gtg gag agc aag gca aag 288
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85 90 95

aca gat ccg tgc gat tag 306
Thr Asp Pro Cys Asp
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<210> 22
<211> 101
<212> PRT
<213> Human immunodeficiency virus

<400> 22

Met Glu Leu Val Asp Pro Asn Leu Asp Pro Trp Asn His Pro Gly Ser
1 5 10 15

Gln Pro Thr Thr Pro Cys Thr Lys Cys Tyr Cys Lys Arg Cys Cys Phe
20 25 30

His Cys Gln Trp Cys Phe Thr Thr Lys Gly Leu Gly Ile Ser Tyr Gly
35 40 45

Arg Lys Lys Arg Arg Gln Arg Arg Arg Thr Pro Gln Ser Ser Gln Ile
50 55 60

His Gln Asp Pro Val Pro Lys Gln Pro Leu Ser Gln Ala Arg Gly Asn

65 70 75 80

Pro Thr Gly Pro Lys Glu Ser Lys Lys Glu Val Glu Ser Lys Ala Lys
85 90 95

Thr Asp Pro Cys Asp
100

<210> 23
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<220>
<221> CDS
<222> (1)..(306)

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Met Asp Pro Val Asp Pro Asn Leu Glu Pro Trp Asn His Pro Gly Ser
1 5 10 15

cag cct aaa act ccg tgt aac aaa tgt tat tgt aaa atg tgt tgc tgg 96
Gln Pro Lys Thr Pro Cys Asn Lys Cys Tyr Cys Lys Met Cys Cys Trp
20 25 30

cat tgt caa gtt tgc ttt ctg aac aaa ggc tta ggc atc tcc tat ggc 144
His Cys Gln Val Cys Phe Leu Asn Lys Gly Leu Gly Ile Ser Tyr Gly
35 40 45

agg aag aag cgg aag cac cga cga gga act cct cag agc agt aag gat 192
Arg Lys Lys Arg Lys His Arg Arg Gly Thr Pro Gln Ser Ser Lys Asp
50 55 60

cat caa aat cct gta cca aag caa ccc tta ccc acc acc aga ggg aac 240
His Gln Asn Pro Val Pro Lys Gln Pro Leu Pro Thr Thr Arg Gly Asn
65 70 75 80

ccg aca ggc ccg aag gaa tcg aag aag gag gtg gag agc aag aca gag 288
Pro Thr Gly Pro Lys Glu Ser Lys Lys Glu Val Glu Ser Lys Thr Glu
85 90 95

aca gat cca ttc gat tag 306
Thr Asp Pro Phe Asp
100

<210> 24
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<212> PRT
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<400> 24

Met Asp Pro Val Asp Pro Asn Leu Glu Pro Trp Asn His Pro Gly Ser
1 5 10 15

Gln Pro Lys Thr Pro Cys Asn Lys Cys Tyr Cys Lys Met Cys Cys Trp

```
            20                  25                  30

His Cys Gln Val Cys Phe Leu Asn Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

Arg Lys Lys Arg Lys His Arg Arg Gly Thr Pro Gln Ser Ser Lys Asp
    50                  55                  60

His Gln Asn Pro Val Pro Lys Gln Pro Leu Pro Thr Thr Arg Gly Asn
65                  70                  75                  80

Pro Thr Gly Pro Lys Glu Ser Lys Lys Glu Val Glu Ser Lys Thr Glu
                85                  90                  95

Thr Asp Pro Phe Asp
            100
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<210> 25
<211> 261
<212> DNA
<213> Human immunodeficiency virus

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<222> (1)..(261)

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Met Asp Pro Val Asp Pro Asn Gln Glu Pro Trp Asn His Pro Gly Ser
1               5                   10                  15

cag cct aaa act gct tgt aac aat tgt tat tgt aaa aag tgc tgc tat      96
Gln Pro Lys Thr Ala Cys Asn Asn Cys Tyr Cys Lys Lys Cys Cys Tyr
            20                  25                  30

cat tgc caa ttg tgc ttt tta aag aaa ggc tta ggc att tcc tat ggc     144
His Cys Gln Leu Cys Phe Leu Lys Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

agg aag aag cgg agc cag cga cga gga act cct gca agt ttg caa gat     192
Arg Lys Lys Arg Ser Gln Arg Arg Gly Thr Pro Ala Ser Leu Gln Asp
    50                  55                  60

cat caa aat cct ata cca aag caa ccc tta tcc cga acc cgc ggg gac     240
His Gln Asn Pro Ile Pro Lys Gln Pro Leu Ser Arg Thr Arg Gly Asp
65                  70                  75                  80

ccg aca ggc ccg aag gaa tag                                        261
Pro Thr Gly Pro Lys Glu
                85
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<210> 26
<211> 86
<212> PRT
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<400> 26

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Gln Pro Lys Thr Ala Cys Asn Asn Cys Tyr Cys Lys Lys Cys Cys Tyr
            20                  25                  30

His Cys Gln Leu Cys Phe Leu Lys Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

Arg Lys Lys Arg Ser Gln Arg Arg Gly Thr Pro Ala Ser Leu Gln Asp
    50                  55                  60

His Gln Asn Pro Ile Pro Lys Gln Pro Leu Ser Arg Thr Arg Gly Asp
65                  70                  75                  80

Pro Thr Gly Pro Lys Glu
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1               5                  10                  15

cag cct aca act gct tgt agc aag tgt tac tgt aaa ata tgt tgc tgg      96
Gln Pro Thr Thr Ala Cys Ser Lys Cys Tyr Cys Lys Ile Cys Cys Trp
            20                  25                  30

cat tgc caa cta tgc ttt ctg aaa aaa ggc tta ggc atc tcc tat ggc     144
His Cys Gln Leu Cys Phe Leu Lys Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

agg aag aag cgg aag cac cga cga gga act cct cag agc agt aag gat     192
Arg Lys Lys Arg Lys His Arg Arg Gly Thr Pro Gln Ser Ser Lys Asp
    50                  55                  60

cat caa aat cct ata cca gag caa ccc cta ccc atc atc aga ggg aac     240
His Gln Asn Pro Ile Pro Glu Gln Pro Leu Pro Ile Ile Arg Gly Asn
65                  70                  75                  80

ccg aca gac ccg aaa gaa tcg aag aag gag gtg gcg agc aag gca gag     288
Pro Thr Asp Pro Lys Glu Ser Lys Lys Glu Val Ala Ser Lys Ala Glu
                85                  90                  95

aca gat ccg tgc gat tag                                            306
Thr Asp Pro Cys Asp
            100
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<210> 28
<211> 101
<212> PRT
<213> Human immunodeficiency virus

<400> 28

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Gln Pro Thr Thr Ala Cys Ser Lys Cys Tyr Cys Lys Ile Cys Cys Trp
            20                  25                  30

His Cys Gln Leu Cys Phe Leu Lys Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

Arg Lys Lys Arg Lys His Arg Arg Gly Thr Pro Gln Ser Ser Lys Asp
    50                  55                  60

His Gln Asn Pro Ile Pro Glu Gln Pro Leu Pro Ile Ile Arg Gly Asn
65                  70                  75                  80

Pro Thr Asp Pro Lys Glu Ser Lys Lys Glu Val Ala Ser Lys Ala Glu
                85                  90                  95

Thr Asp Pro Cys Asp
            100
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<220>
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<222> (1)..(306)

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1               5                  10                  15

cag cct aca act gct tgt agc aat tgt tac tgt aaa atg tgc tgc tgg      96
Gln Pro Thr Thr Ala Cys Ser Asn Cys Tyr Cys Lys Met Cys Cys Trp
            20                  25                  30

cat tgc caa ttg tgc ttt ctg aac aag ggc tta ggc atc tcc tat ggc     144
His Cys Gln Leu Cys Phe Leu Asn Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

agg aag aag cgg aga cgc cga cga gga act cct cag agc cgt cag gat     192
Arg Lys Lys Arg Arg Arg Arg Arg Gly Thr Pro Gln Ser Arg Gln Asp
    50                  55                  60

cat caa aat cct gta cca aag caa ccc tta ccc acc acc aga ggg aac     240
His Gln Asn Pro Val Pro Lys Gln Pro Leu Pro Thr Thr Arg Gly Asn
65                  70                  75                  80
```

ccg aca ggc ccg aaa gaa tcg aag aag gag gtg gcg agc aag aca gag 288
Pro Thr Gly Pro Lys Glu Ser Lys Lys Glu Val Ala Ser Lys Thr Glu
85 90 95

aca gat ccg tgc gat tag 306
Thr Asp Pro Cys Asp
100

<210> 30
<211> 101
<212> PRT
<213> Human immunodeficiency virus

<400> 30

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1 5 10 15

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20 25 30

His Cys Gln Leu Cys Phe Leu Asn Lys Gly Leu Gly Ile Ser Tyr Gly
35 40 45

Arg Lys Lys Arg Arg Arg Arg Arg Gly Thr Pro Gln Ser Arg Gln Asp
50 55 60

His Gln Asn Pro Val Pro Lys Gln Pro Leu Pro Thr Thr Arg Gly Asn
65 70 75 80

Pro Thr Gly Pro Lys Glu Ser Lys Lys Glu Val Ala Ser Lys Thr Glu
85 90 95

Thr Asp Pro Cys Asp
100

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<213> Human immunodeficiency virus

<220>
<221> CDS
<222> (1)..(348)

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1 5 10 15

cag ccc cag acc cct tgt aat aag tgc tat tgc aaa aga tgc tgc tat 96
Gln Pro Gln Thr Pro Cys Asn Lys Cys Tyr Cys Lys Arg Cys Cys Tyr
20 25 30

cat tgc tat gtt tgt ttt gca agc aag ggt ttg gga atc tcc tat ggc 144
His Cys Tyr Val Cys Phe Ala Ser Lys Gly Leu Gly Ile Ser Tyr Gly
35 40 45

```
 agg aag aag cga cgg aga cca gcc gct gct gcg agc cat cca gat aat      192
Arg Lys Lys Arg Arg Arg Pro Ala Ala Ala Ala Ser His Pro Asp Asn
    50                  55                  60

caa gat cct gta cca gag caa ccc cca tcc atc acc aac agg aag cag      240
Gln Asp Pro Val Pro Glu Gln Pro Pro Ser Ile Thr Asn Arg Lys Gln
65                  70                  75                  80

aaa cgc cag gag gaa cag gag aag gag gtg gag aag gag aca ggc cca      288
Lys Arg Gln Glu Glu Gln Glu Lys Glu Val Glu Lys Glu Thr Gly Pro
                85                  90                  95

ggt gga tac cct cgc cgc aag gat tct tgc cac tgt tgt aca cgg acc      336
Gly Gly Tyr Pro Arg Arg Lys Asp Ser Cys His Cys Cys Thr Arg Thr
            100                 105                 110

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Ser Gly Gln
        115
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<210> 32
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<213> Human immunodeficiency virus

<400> 32

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            20                  25                  30

His Cys Tyr Val Cys Phe Ala Ser Lys Gly Leu Gly Ile Ser Tyr Gly
        35                  40                  45

Arg Lys Lys Arg Arg Arg Pro Ala Ala Ala Ala Ser His Pro Asp Asn
    50                  55                  60

Gln Asp Pro Val Pro Glu Gln Pro Pro Ser Ile Thr Asn Arg Lys Gln
65                  70                  75                  80

Lys Arg Gln Glu Glu Gln Glu Lys Glu Val Glu Lys Glu Thr Gly Pro
                85                  90                  95

Gly Gly Tyr Pro Arg Arg Lys Asp Ser Cys His Cys Cys Thr Arg Thr
            100                 105                 110

Ser Gly Gln
        115
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<210> 33
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<213> Human immunodeficiency virus

<400> 33

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1 5 10 15

<210> 34
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<400> 34

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<210> 35
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<400> 35

Trp Lys His Pro Gly Ser Gln Pro Lys Thr Ala Cys Thr Asn Cys
1 5 10 15

<210> 36
<211> 15
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<400> 36

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1 5 10 15

<210> 37
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<400> 37

Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys Gln
1 5 10 15

<210> 38
<211> 15
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<400> 38

Tyr Cys Lys Lys Cys Cys Phe His Cys Gln Val Cys Phe Ile Thr
1 5 10 15

<210> 39
<211> 15
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<400> 39

Cys Phe His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile
1 5 10 15

<210> 40
<211> 15
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<400> 40

Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys
1 5 10 15

<210> 41
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<400> 41

Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys Lys Arg Arg Gln Arg
1 5 10 15

<210> 42
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<400> 42

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1 5 10 15

<210> 43
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<400> 43

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1 5 10 15

<210> 44
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<213> Human immunodeficiency virus

<400> 44

Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln Val Ser Leu Ser
1 5 10 15

<210> 45
<211> 15
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<400> 45

Gly Ser Gln Thr His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser
1 5 10 15

<210> 46
<211> 15
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<400> 46

Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
1 5 10 15

<210> 47
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<400> 47

Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr Gly Pro Lys
1 5 10 15

<210> 48
<211> 15
<212> PRT
<213> Human immunodeficiency virus

<400> 48

Gln Ser Arg Gly Asp Pro Thr Gly Pro Lys Glu Gln Lys Lys Lys
1 5 10 15

CLAIMS

1. A microparticle comprising:

(a) a core which comprises a water insoluble polymer or copolymer, and

(b) a shell which comprises a hydrophilic polymer or copolymer and functional groups which are ionic or ionisable;

said microparticle having a disease-associated antigen adsorbed at the external surface.

2. A microparticle according to claim 1, wherein the disease-associated antigen is a microbial antigen or a cancer-associated antigen.

3. A microparticle according to claim 1 or 2, wherein the water insoluble polymer is poly(styrene).

4. A microparticle according to claim 1 or 2, wherein the water insoluble polymer is poly(methylmethacrylate).

5. A microparticle according to any one of the preceding claims, wherein the hydrophilic polymer is hemisuccinated polyvinylalcohol.

6. A microparticle according to any one of claims 1 to 4, wherein the hydrophilic copolymer is Eudragit® L100-55 (a copolymer of methyacrylic acid and ethyl acrylate).

7. A microparticle according to any of the preceding claims, wherein the particle has a maximum size of from 0.1 to 10μm.

8. A microparticle according to any of the preceding claims, wherein the antigen is a human immunodeficiency virus-1 (HIV-1) antigen.

9. A microparticle according to claim 8, wherein the antigen is HIV-1 Tat protein (SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32) or an immunogenic fragment thereof.

10. A method of production of a microparticle according to any one of the preceding claims, said method comprising:

(a) polymerizing one or more water insoluble monomers in the presence of one or more hydrophilic polymer by dispersion polymerization to form microparticles; and

(b) adsorbing a disease-associated antigen at the external surface of said microparticles.

11. A pharmaceutical composition comprising a microparticle according

to any one of claims 1 to 9 and a pharmaceutically acceptable excipient

12. A method of generating an immune response in an individual, said method comprising administering a microparticle according to any one of claims 1 to 9 or a pharmaceutical composition according to claim 11 in a therapeutically effective amount.

13. A method of preventing or treating HIV infection or AIDS, said method comprising administering a microparticle according to claim 8 or 9 in a therapeutically effective amount.

14. A microparticle according to any one of claims 1 to 9 or a pharmaceutical composition according to claim 11 for use in a method of treatment of the human or animal body by therapy or diagnosis.

15. Use of a microparticle according to any one of claims 1 to 9 for the manufacture of a medicament for generating an immune response in an individual.

16. Use of a microparticle according to claim 8 or 9 for the manufacture of a medicament for preventing or treating HIV infection or AIDS.

ABSTRACT

USE OF MICROPARTICLES FOR PROTEIN DELIVERY

The invention relates to microparticles that may be used for antigen delivery and vaccine immunization strategies. The invention in particular relates to microparticles that are useful in the prophylaxis and treatment of human immunodeficiency virus (HIV) infections.

Figure 1

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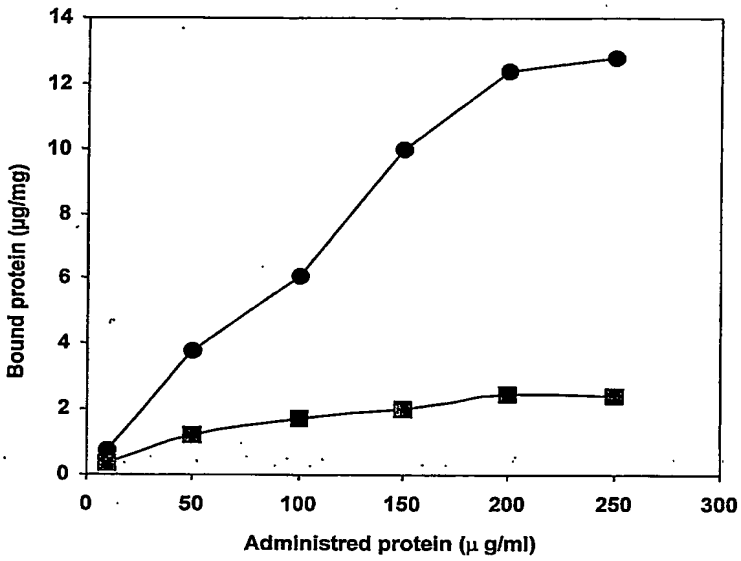
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12
10
8
6
4
2
0
Bound protein (µg/mg)
0
50
100
150
200
250
300
Administred protein (µ g/ml)

B

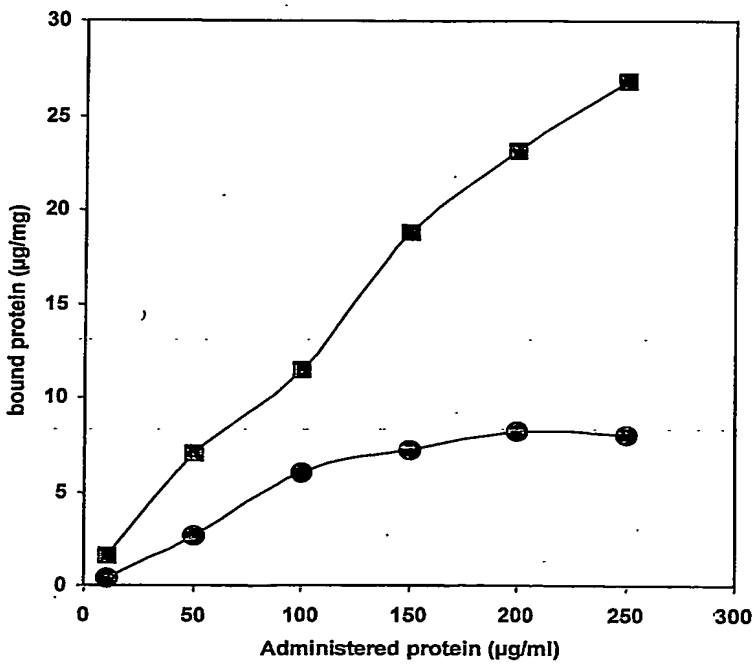

30
25
20
15
10
5
0
bound protein (µg/mg)
0
50
100
150
200
250
300
Administered protein (µg/ml)

Figure 2

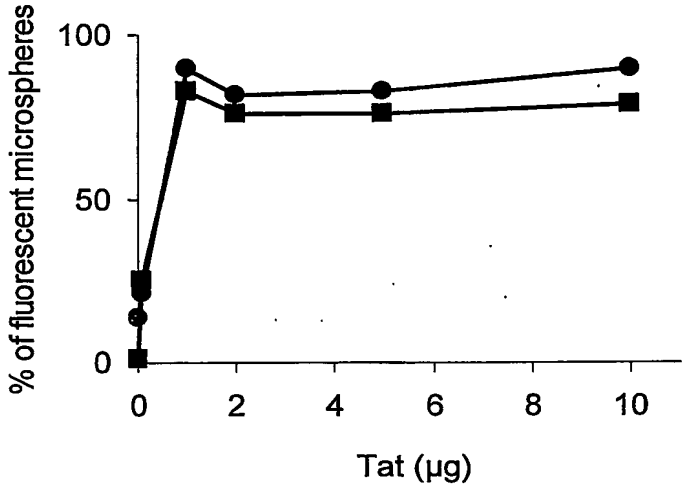
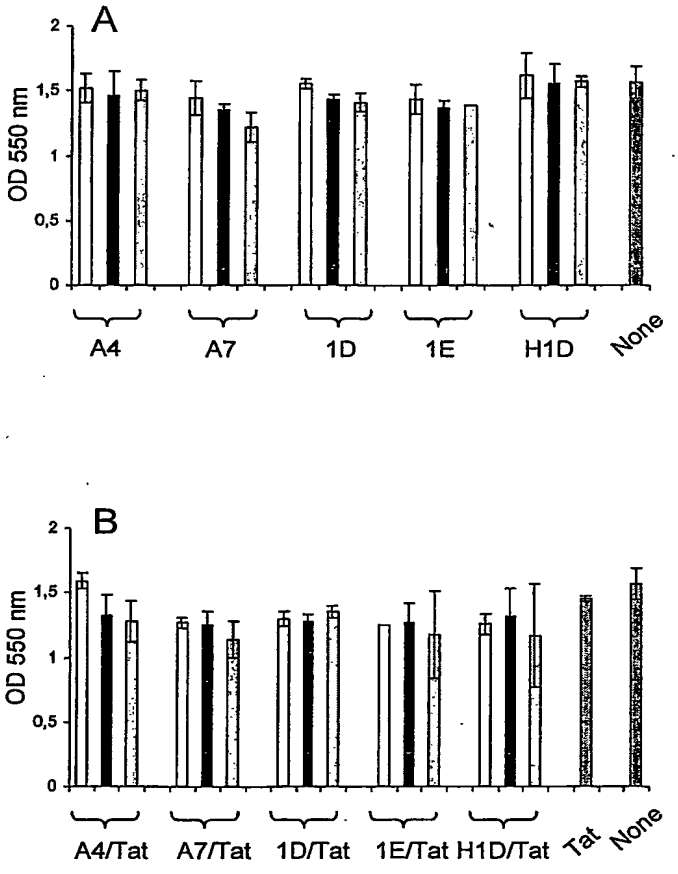


Figure 3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OD 550 nm
2
1,5
1
0,5
0
A4
A7
1D
1E
H1D
None
B
OD 550 nm
2
1,5
1
0,5
0
A4/Tat
A7/Tat
1D/Tat
1E/Tat
H1D/Tat
Tat
None

Figure 4

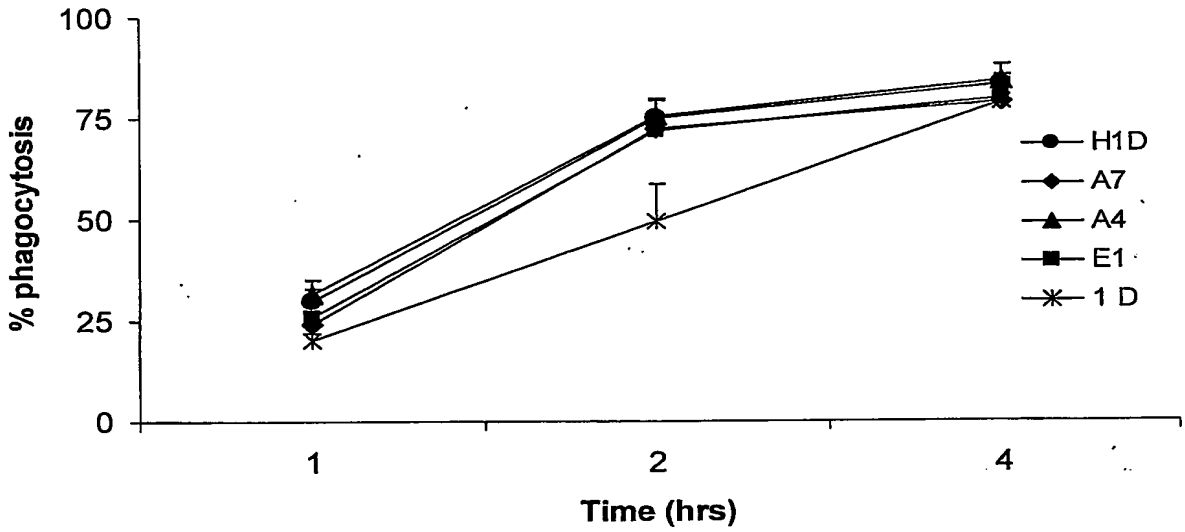
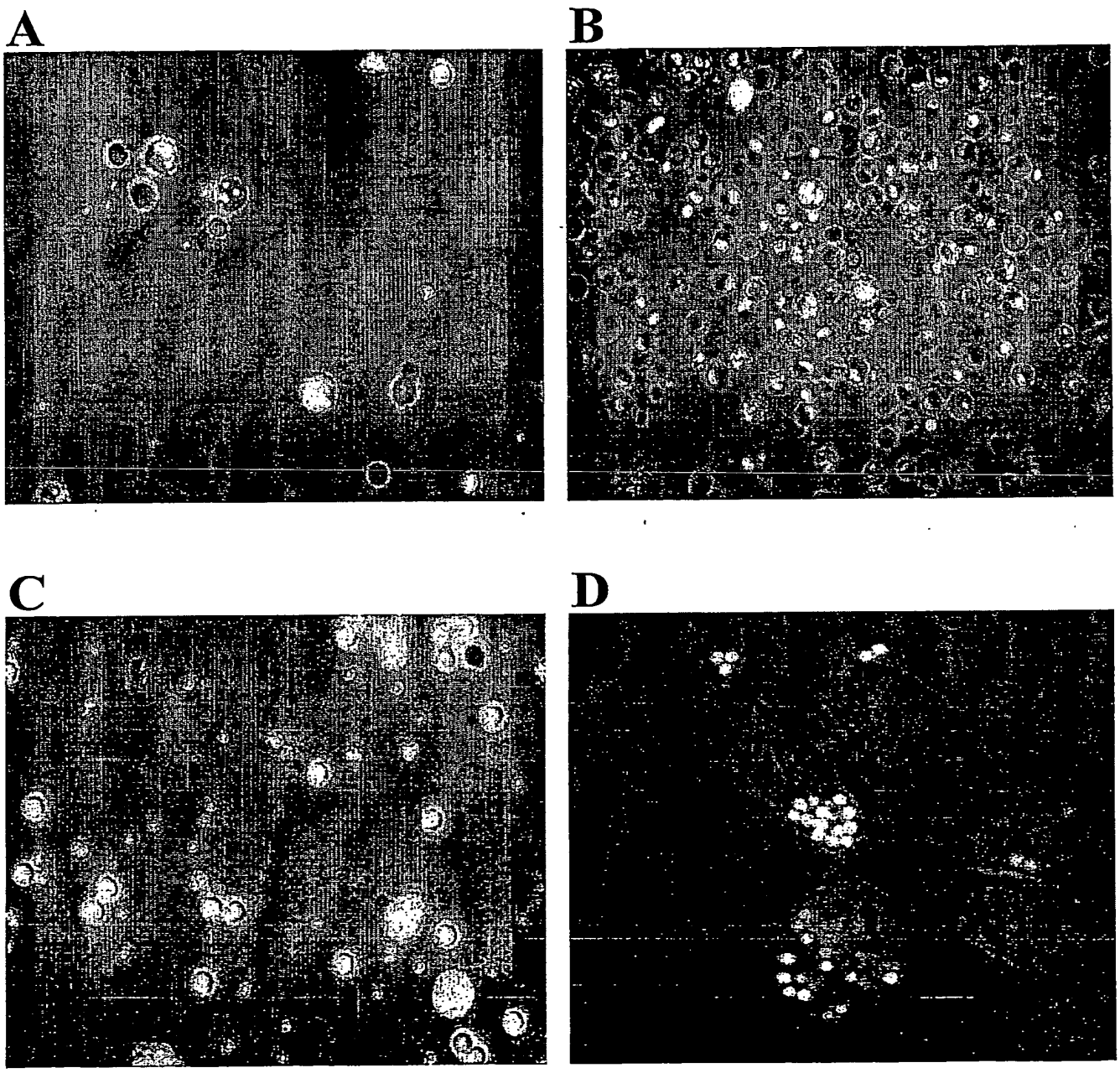


Figure 5



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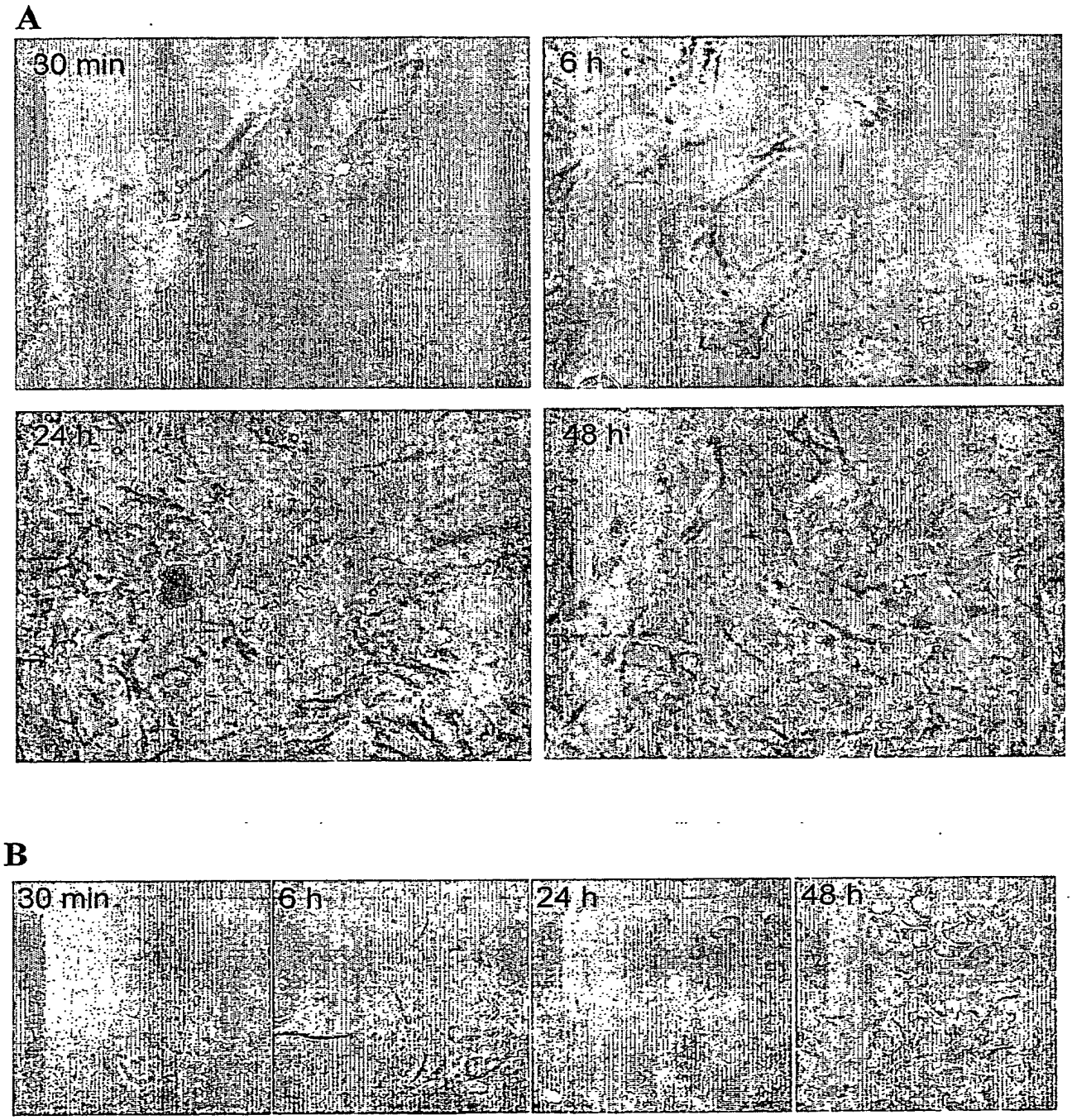

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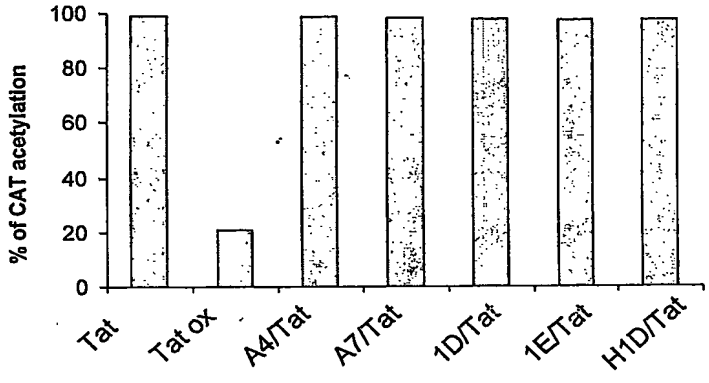
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80
60
40
20
0
Tat
Tat ox
A4/Tat
A7/Tat
1D/Tat
1E/Tat
H1D/Tat

Figure 8

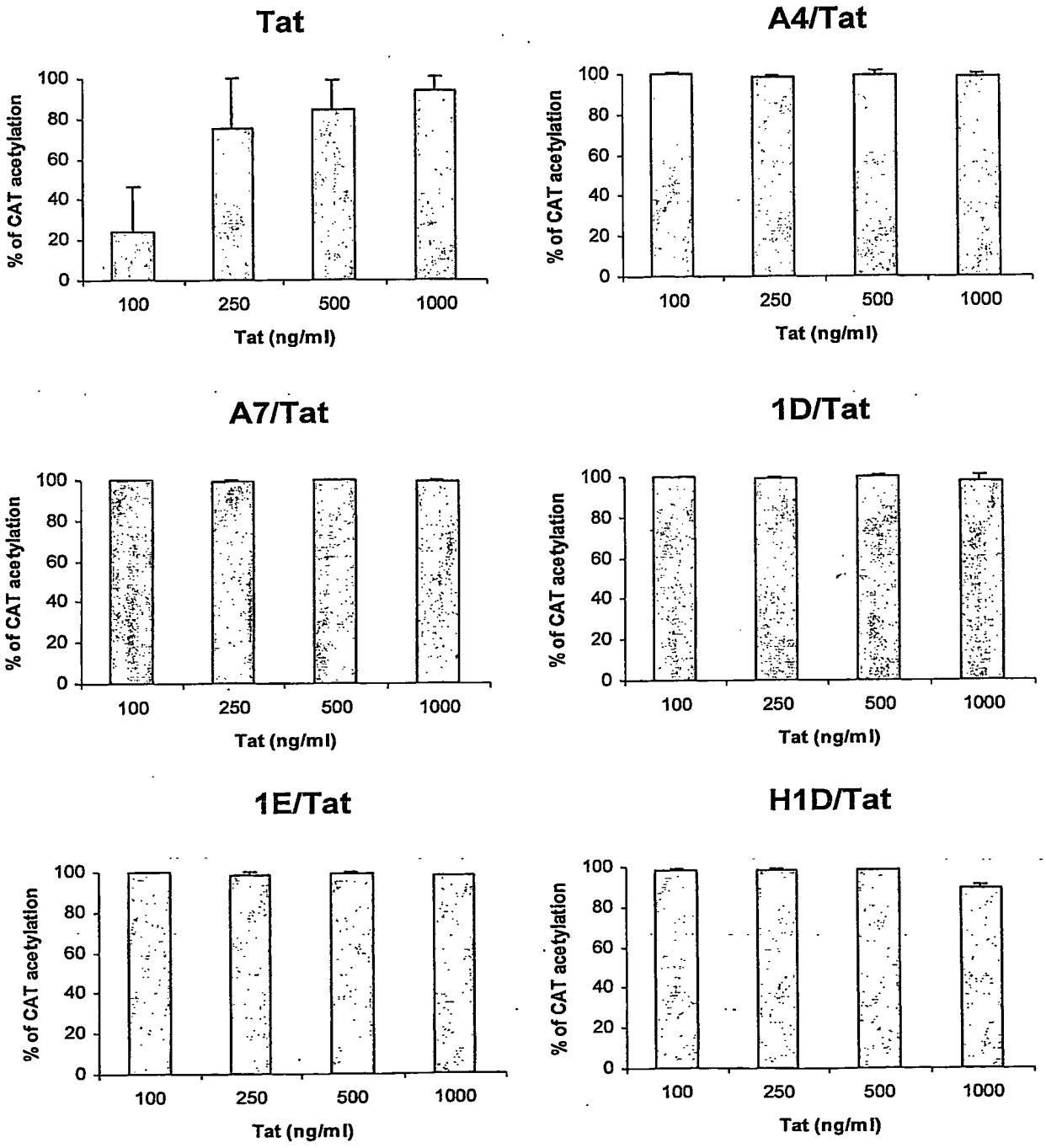
Tat
A4/Tat
A7/Tat
1D/Tat
1E/Tat
H1D/Tat
% of CAT acetylation
0
20
40
60
80
100
100
250
500
1000
Tat (ng/ml)

Figure 9

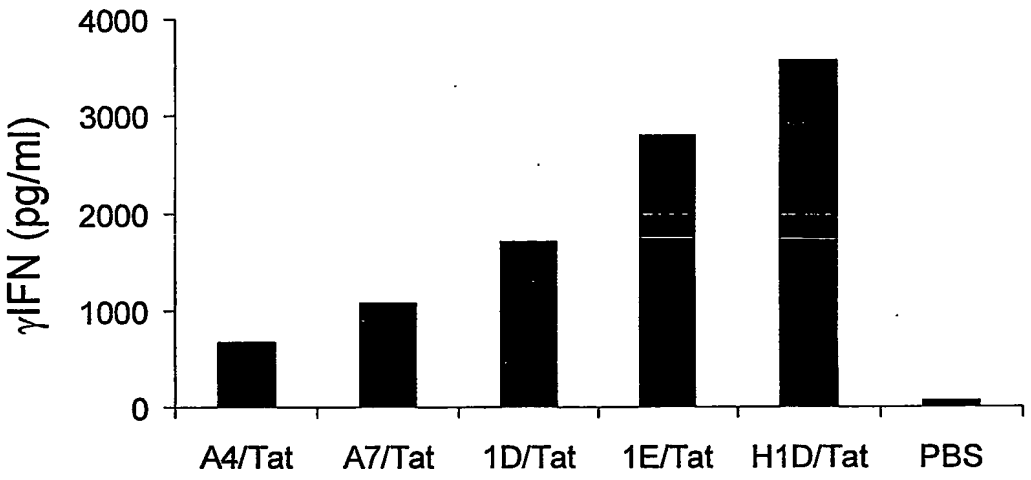
4000
3000
2000
1000
0
γIFN (pg/ml)
A4/Tat
A7/Tat
1D/Tat
1E/Tat
H1D/Tat
PBS

Figure 10

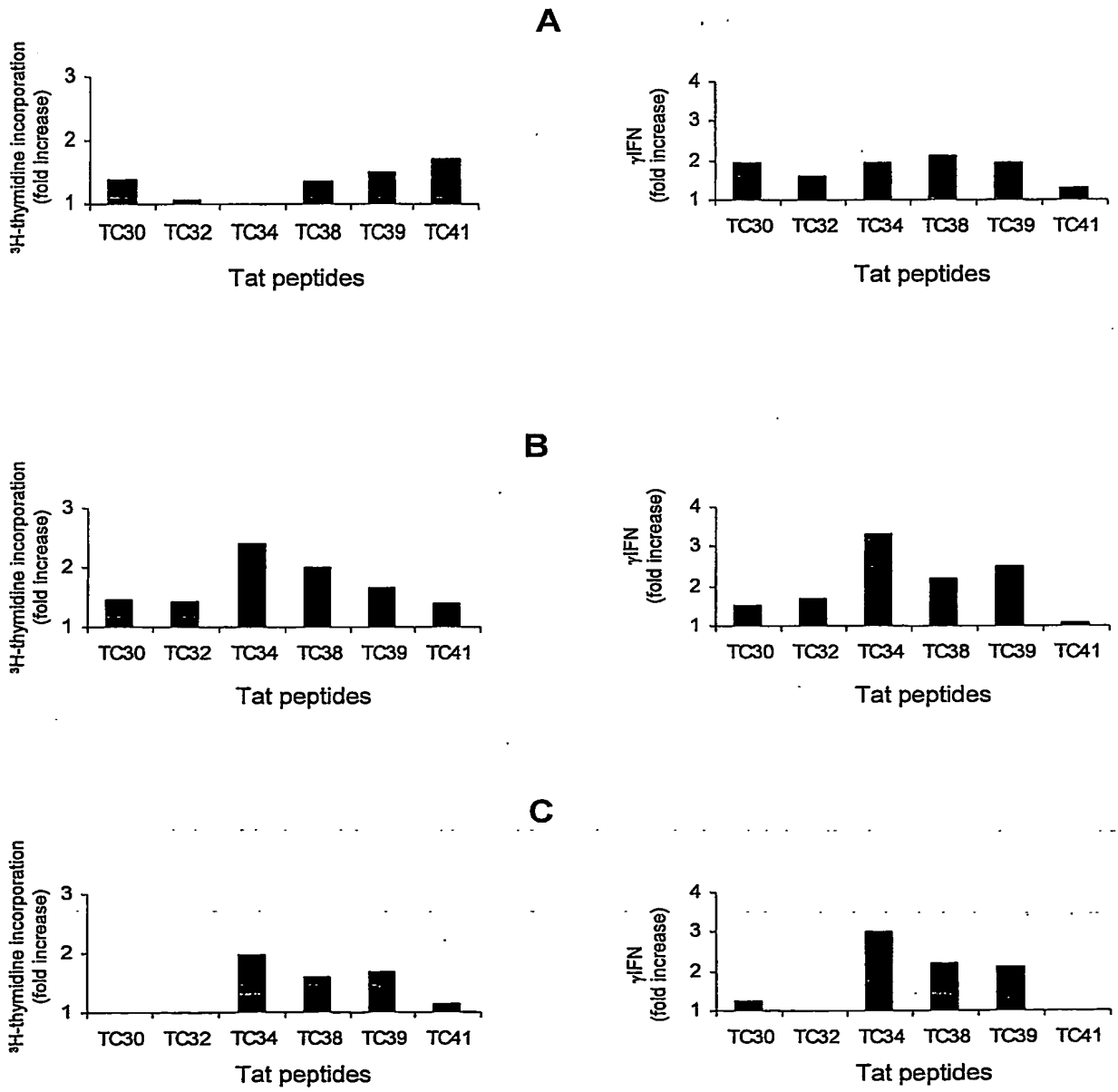
A
^{3}H-thymidine incorporation (fold increase)
3
2
1
TC30 TC32 TC34 TC38 TC39 TC41
Tat peptides
γIFN (fold increase)
4
3
2
1
TC30 TC32 TC34 TC38 TC39 TC41
Tat peptides
B
^{3}H-thymidine incorporation (fold increase)
3
2
1
TC30 TC32 TC34 TC38 TC39 TC41
Tat peptides
γIFN (fold increase)
4
3
2
1
TC30 TC32 TC34 TC38 TC39 TC41
Tat peptides
C
^{3}H-thymidine incorporation (fold increase)
3
2
1
TC30 TC32 TC34 TC38 TC39 TC41
Tat peptides
γIFN (fold increase)
4
3
2
1
TC30 TC32 TC34 TC38 TC39 TC41
Tat peptides

Figure 11